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(54) **MULTIMERIC FORMS OF ANTIMICROBIAL PEPTIDES**

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# Related U.S. Application Data

(63) Continuation-in-part of application No. 12/988,994, filed as application No. PCT/SG2009/000144 on Apr. 21, 2009, now Pat. No. 8,809,262.

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# (58) Field of Classification Search

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See application file for complete search history.

# (57) ABSTRACT

The invention relates to multimeric forms of antimicrobial peptides, for example, defensin peptides. The multimeric forms of defensin peptides possesses antimicrobial activity and may be formulated into antimicrobial compositions, pharmaceutical compositions, eyedrop composition, contact lens solution compositions for coating medical devices and the like. The invention also relates to the use of these multimeric forms of peptides, e.g. multimeric forms of defensin peptides for inhibiting and/or reducing the growth of microorganisms in general, including in a host. The invention further relates to a method of preparing multimers of peptides derived from defensins, for example hBD3. The method includes a composition or combination comprising the multimeric antimicrobial peptides and at least one active pharmaceutical ingredient.

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**18 Claims, 11 Drawing Sheets**

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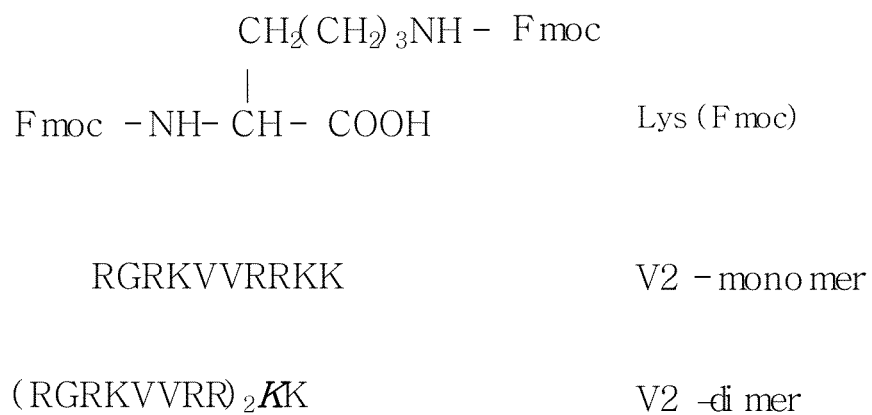
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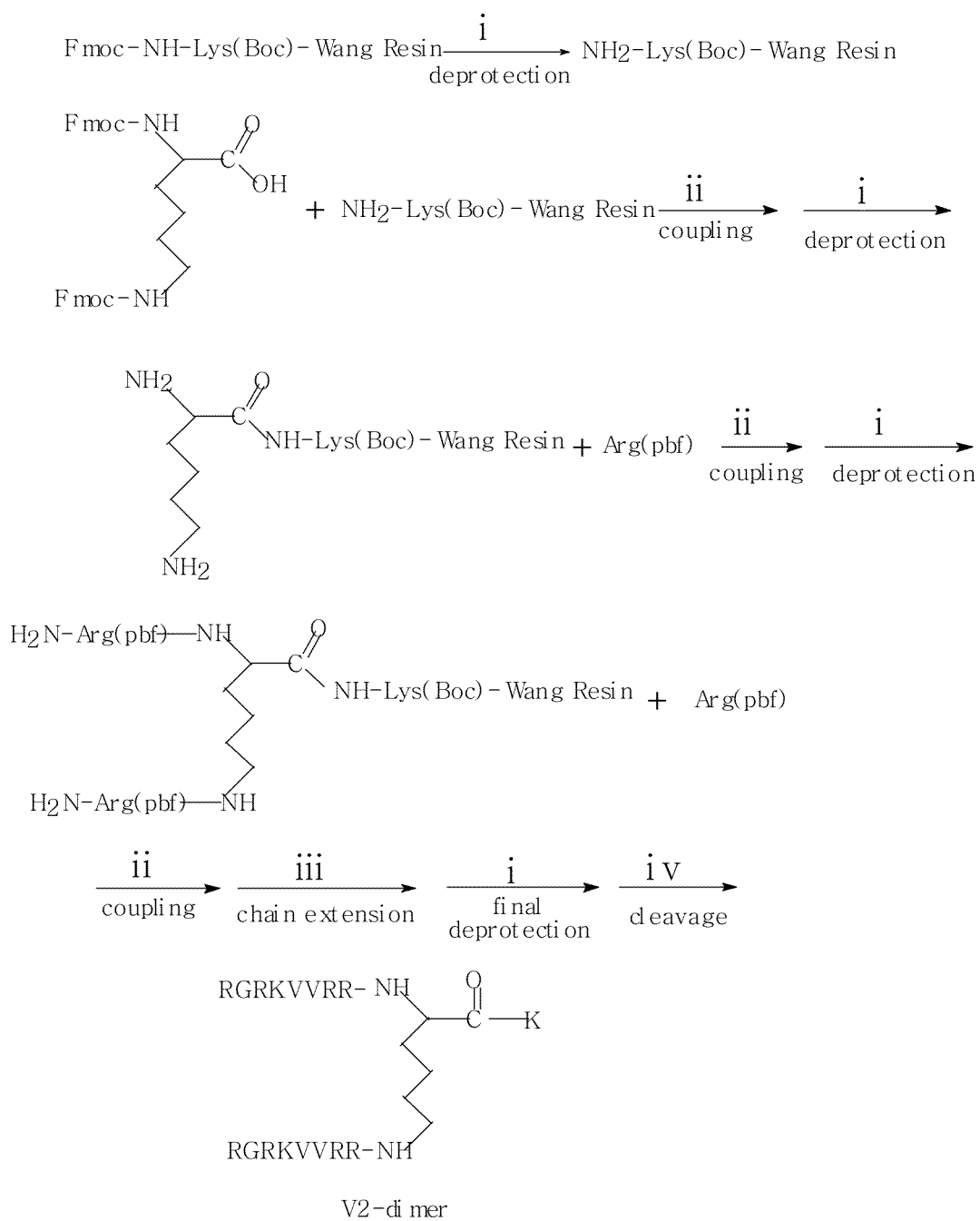
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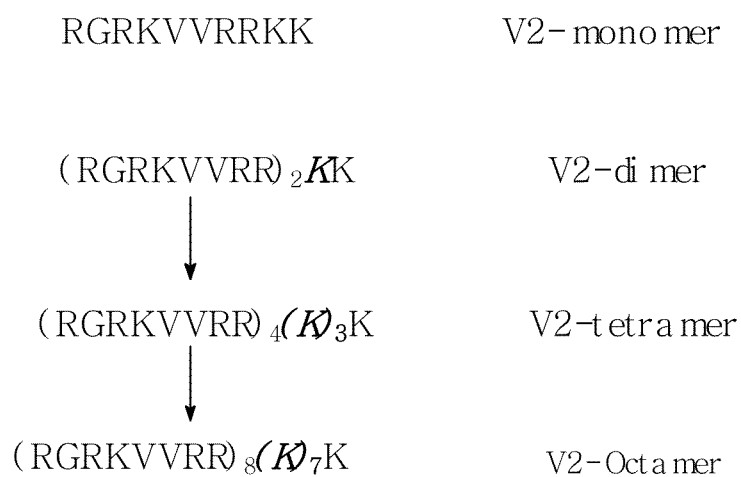
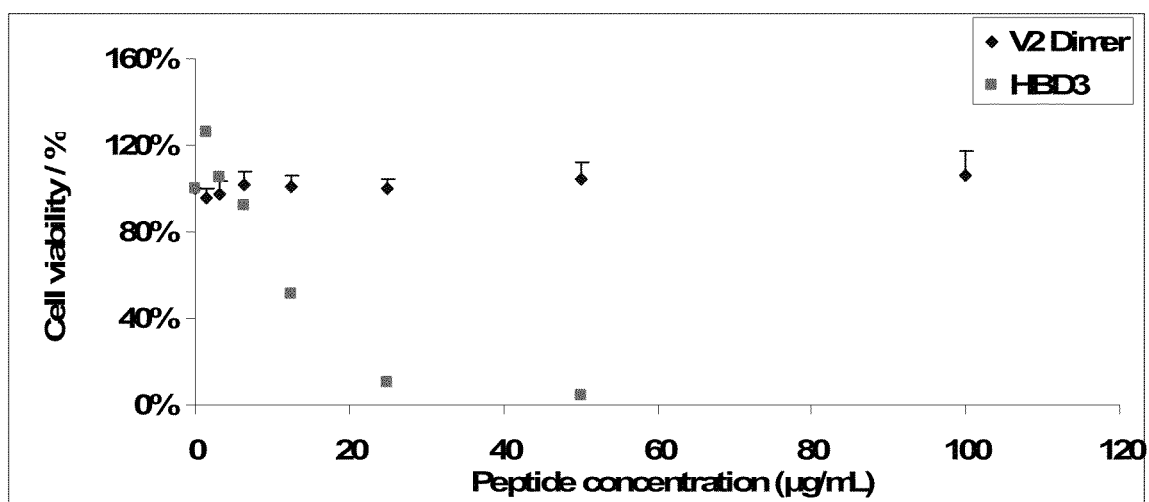
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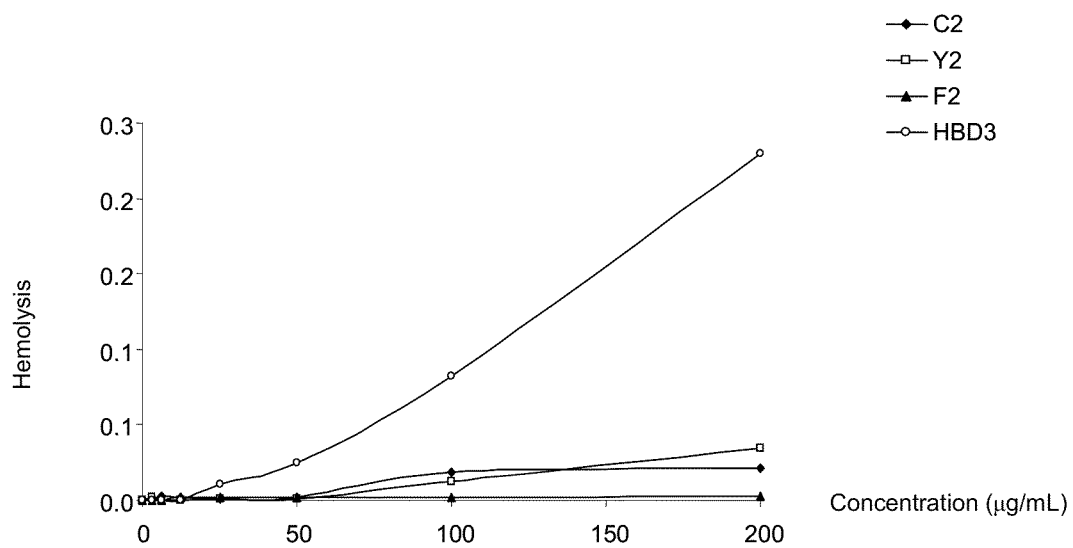
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**Figure 1**

### **Figure 2**



**Figure 3****Figure 4**

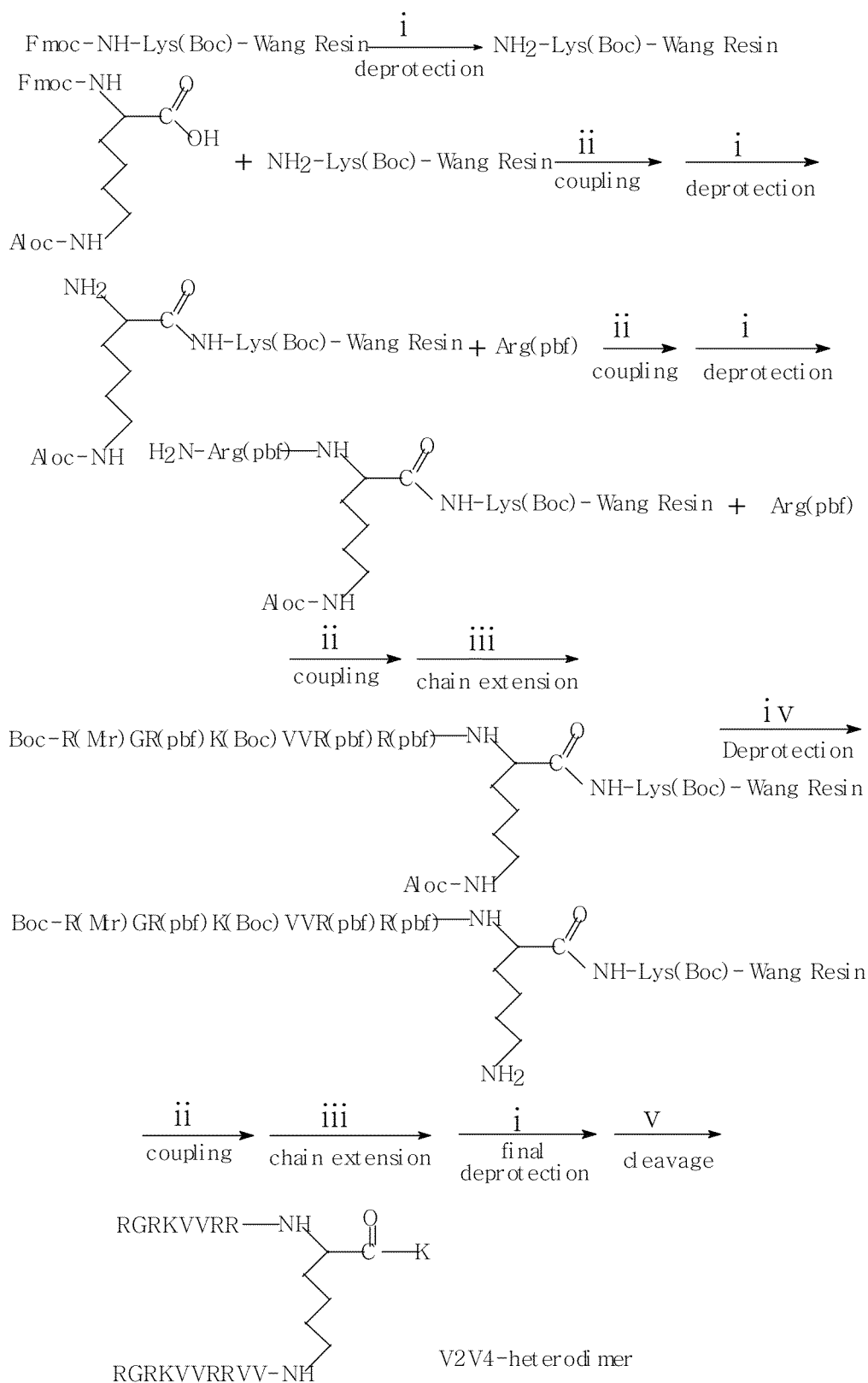
**Figure 5**

Percentage Cell Survival

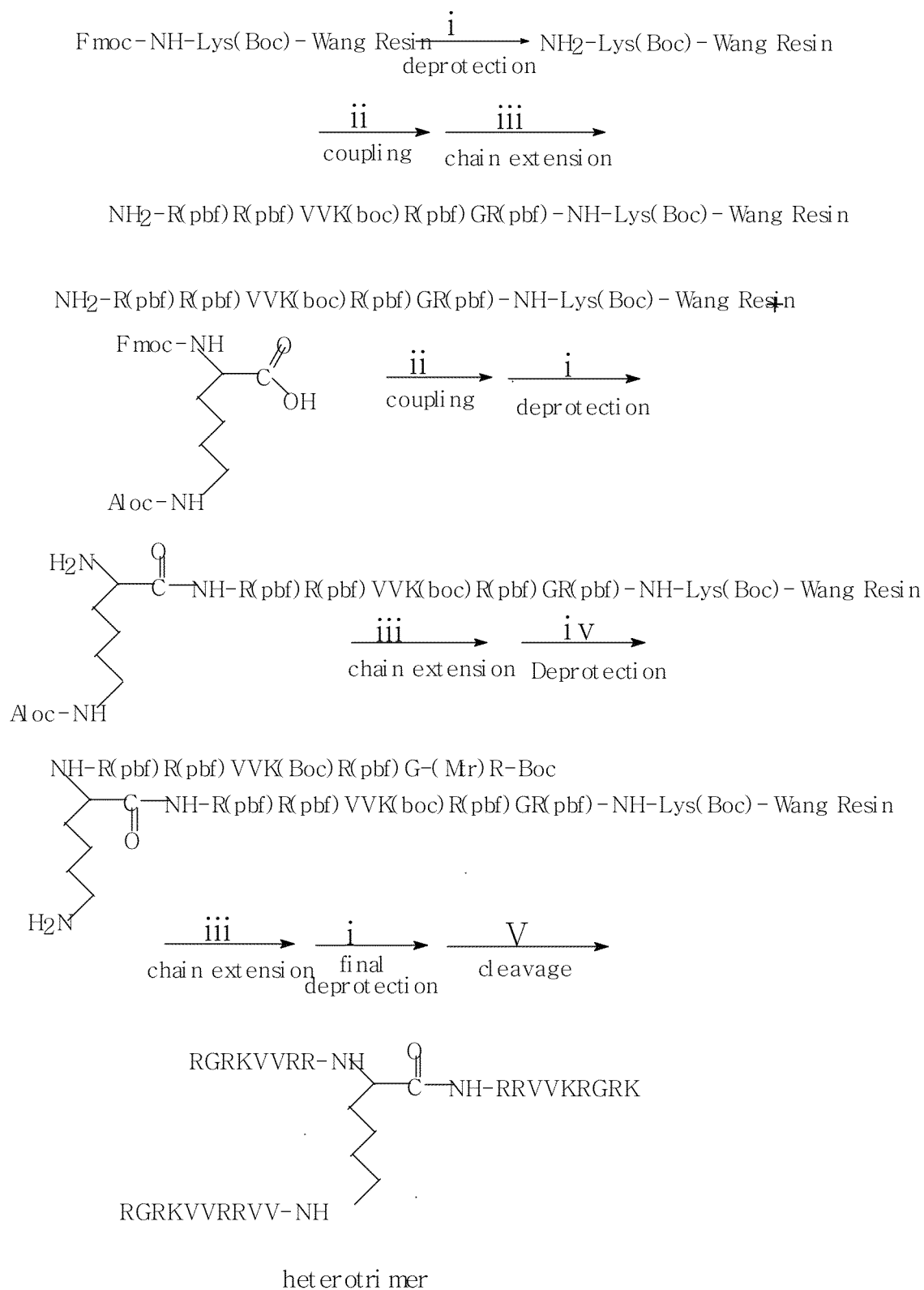
Concentration ratio (ug/ml)

Legend:

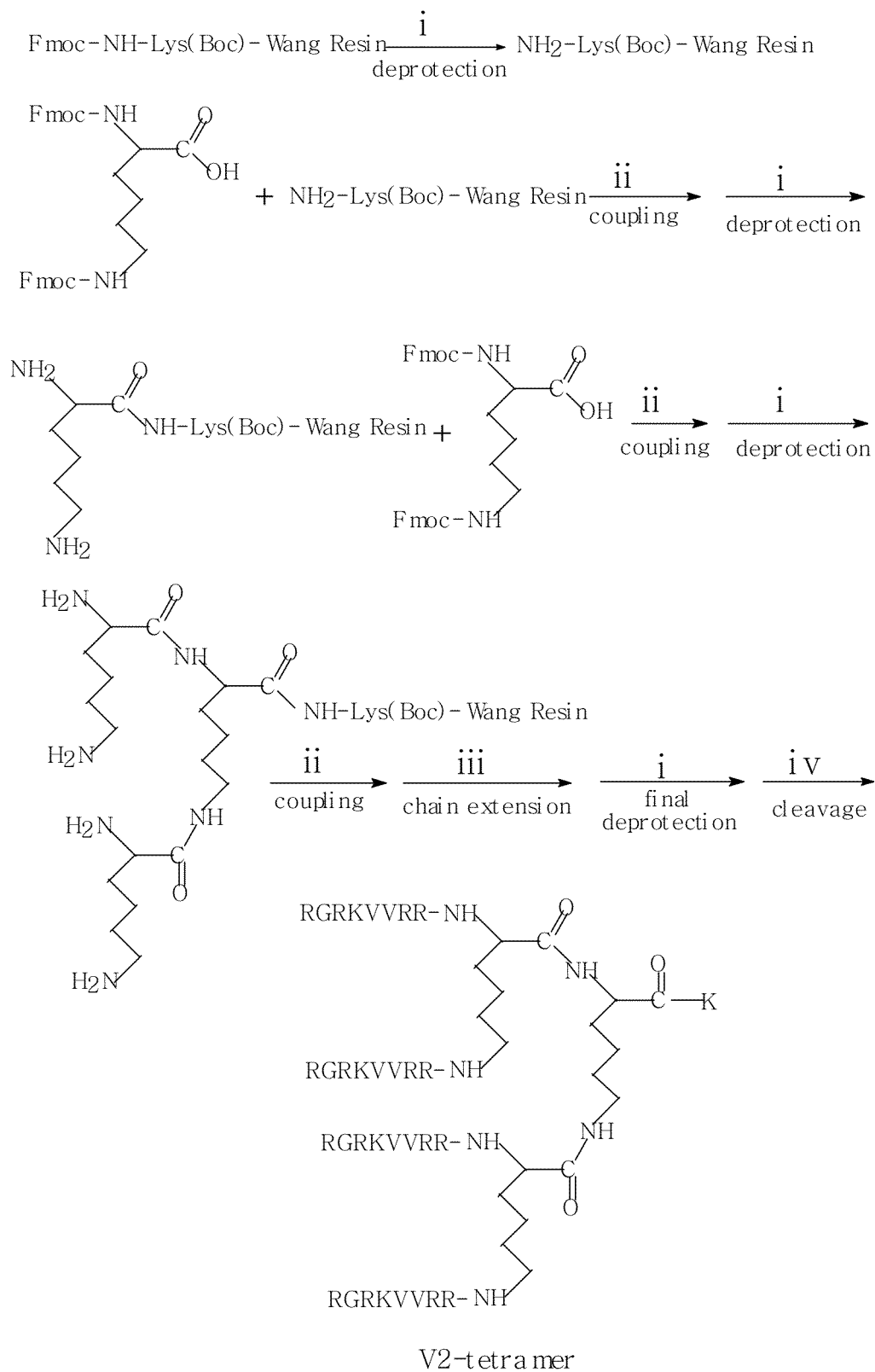
- L2
- v2
- y2
- v2
- F2
- I2
- c2
- H2

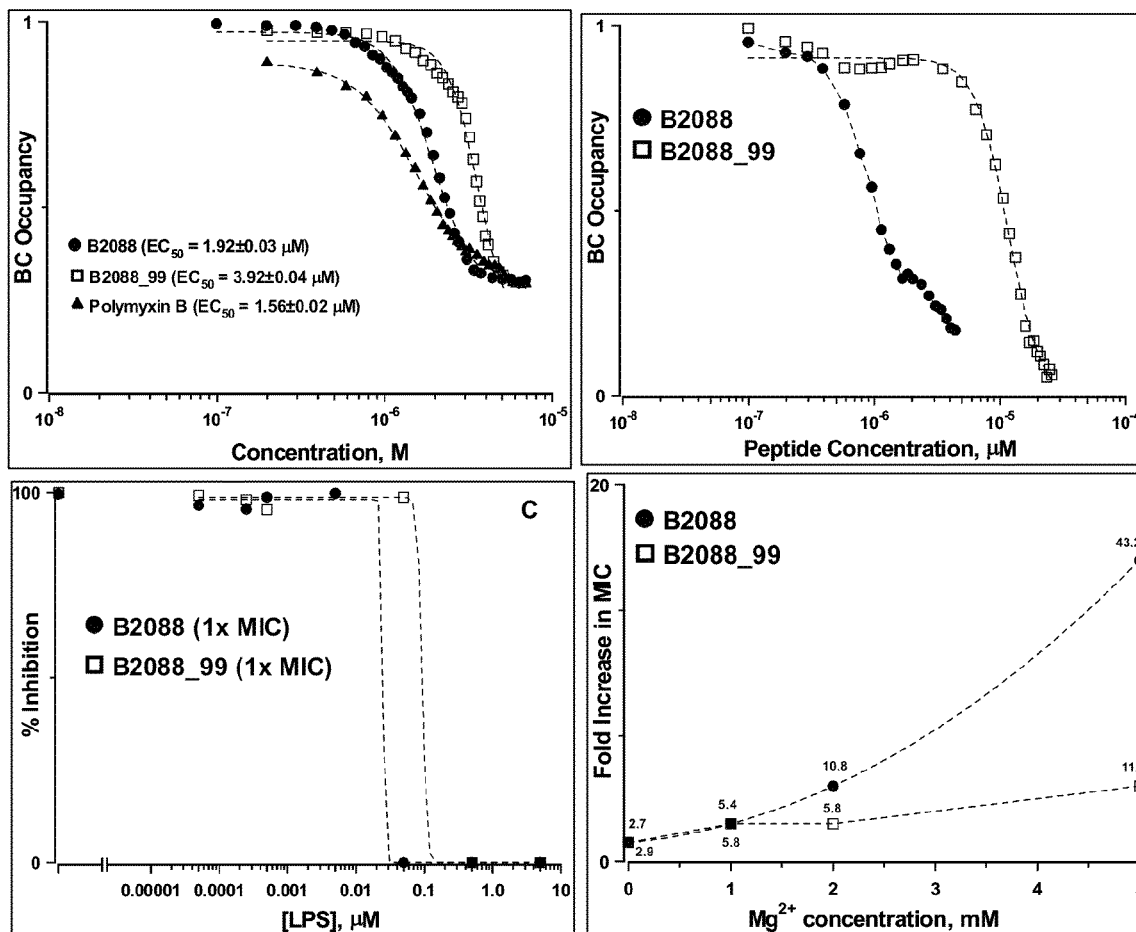
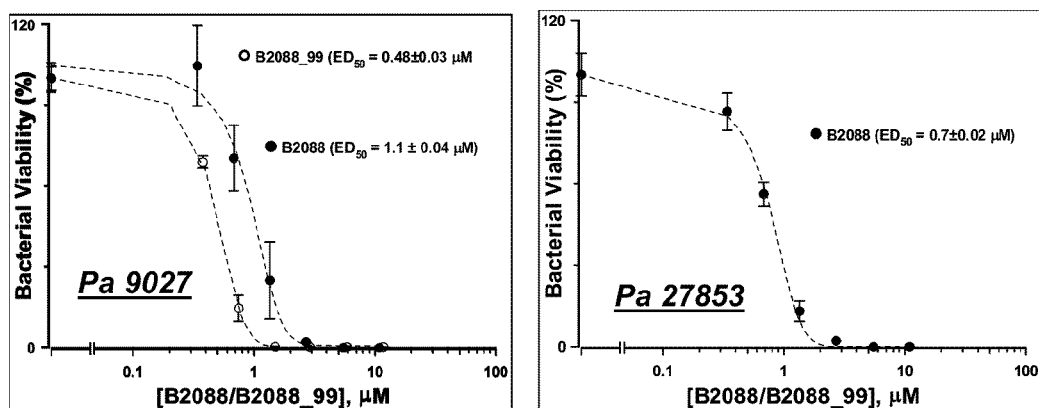
**Figure 7**

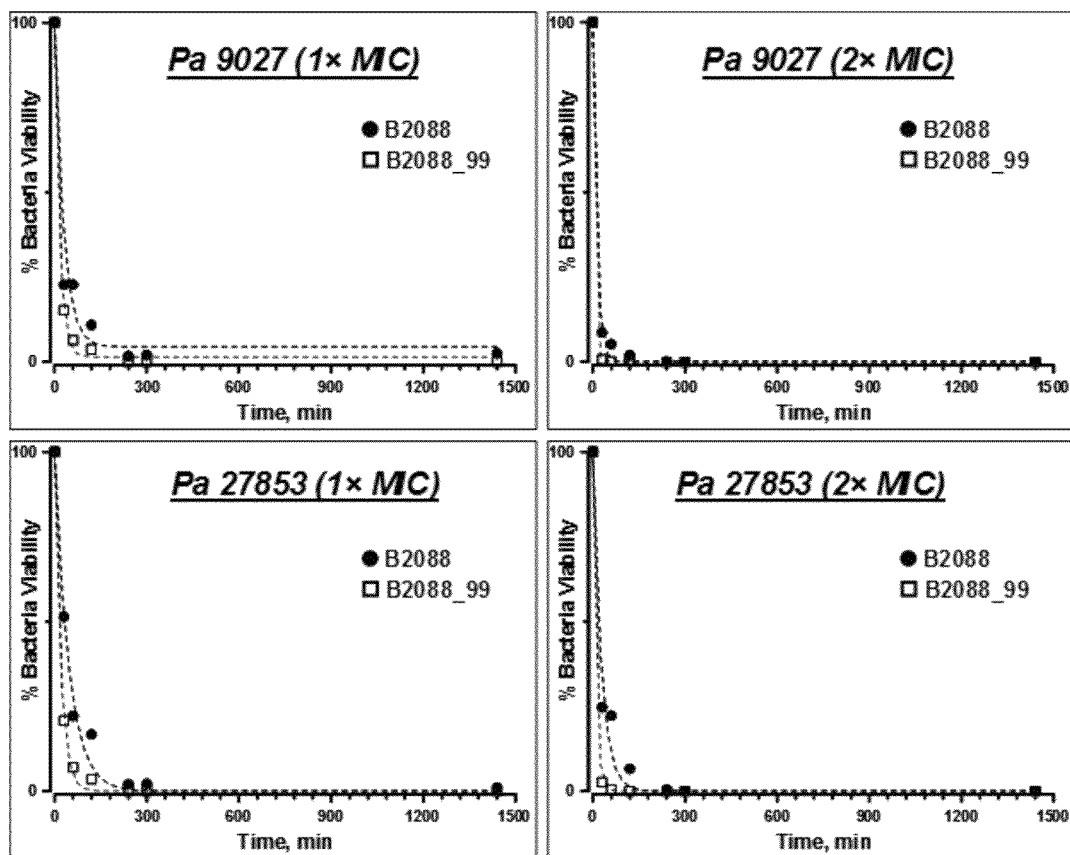
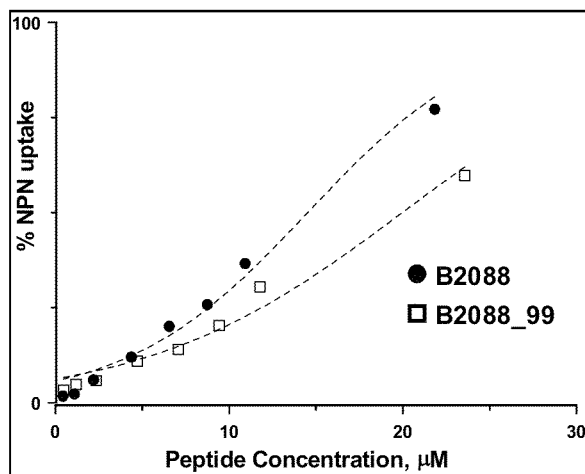


**Figure 8**

### Figure 9

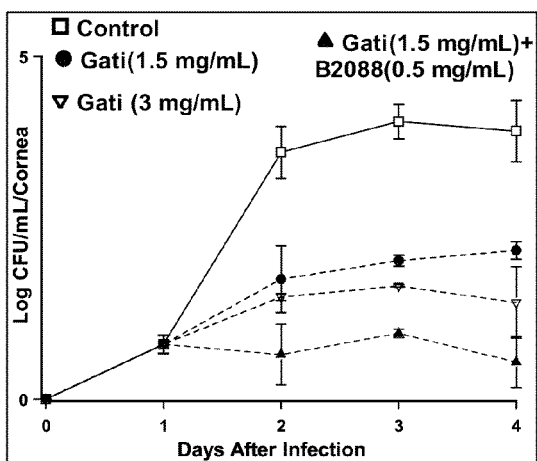


**Figure 10****Figure 11**

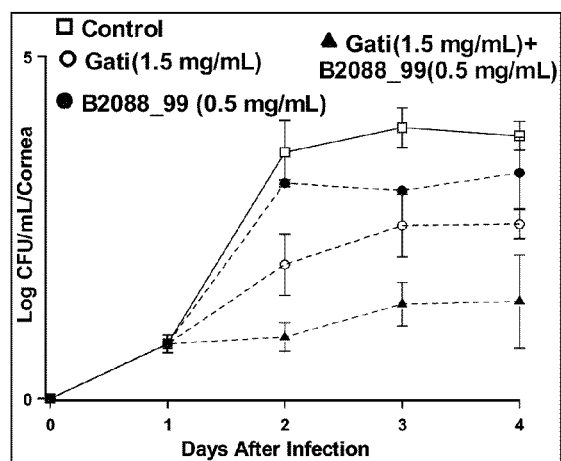
**Figure 12****Figure 13**

**Figure 14**

A



B



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**MULTIMERIC FORMS OF ANTIMICROBIAL PEPTIDES****CROSS REFERENCE TO RELATED APPLICATIONS**

The present application is a continuation-in-part of U.S. Ser. No. 12/988,994 (the US national phase entry of PCT/SG2009/000144 filed on Apr. 21, 2009) filed Oct. 21, 2010, which in turns claims priority from U.S. 61/046,584 filed on Apr. 21, 2008. The whole contents of these three applications are herein incorporated by reference in their entirety.

**CROSS REFERENCE TO SEQUENCE LISTING**

The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (file-name: Sequence\_Listing.txt, date recorded: Nov. 26, 2013, file size 24 kilobytes).

**FIELD OF THE INVENTION**

The present invention relates to novel multimeric forms of peptides with antimicrobial properties. The invention also relates to methods of making these multimers. The invention relates to the use of these multimers for inhibiting the growth of a broad spectrum of microorganisms. The invention further relates to compositions comprising these peptides. The invention also relates to compositions and/or combinations comprising these peptides with at least one active pharmaceutical ingredient, for example, at least one antimicrobial compound.

**BACKGROUND OF THE INVENTION**

Defensins are cationic antimicrobial peptides and are components of the innate immune system. In humans, the alpha defensins are produced by neutrophils or the Paneth cells of the intestinal tract while the beta defensins are produced by epithelial cells. Defensins have broad spectrum anti-microbial properties against gram negative and gram positive bacteria, some fungi as well as enveloped viruses.

The exact mechanism for the anti-microbial properties is not completely understood but the hydrophobicity and the net positive charge of the peptides appear to be important in its interaction and disruption of the microbial cell wall and cell membrane.

Several studies suggest that full length defensins may form dimers at a bacterial membrane but non-covalent forms (Hoover et al., 2000; Hoover et al., 2001; Schibili et al., 2002) and one study suggests that dimerisation may affect antimicrobial properties (Campopiano et al., 2004). However, the properties of these dimers were not studied or characterised in detail.

The development of antibiotic resistance is a challenge in the development of antibiotics. Although the antimicrobial activity of defensins is not completely understood, the possible mode of action of defensins suggests that microbial resistance may develop very slowly or minimally and the potential of using defensins as antimicrobials is promising. However, defensins are also known to have toxicity to host cells, including mammalian cells which may limit their application as antimicrobials.

It is therefore desirable to develop new peptide derivatives of defensins with high antimicrobial activity and low host cell toxicity and new compositions or combinations of these pep-

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tides with at least one other active pharmaceutical ingredient, for example at least one antimicrobial compound.

**SUMMARY OF THE INVENTION**

The present invention relates to isolated multimers derived from hBD3 (SEQ ID NO: 1).

hBD3

(SEQ ID NO: 1)

GIINTLQKYYCRVGRCAVLSCLPKEEQIGKCSRGRKCCRRKK

According to a first aspect, the present invention relates to an isolated multimer of defensin peptides of formula  $(U)_n$ , wherein U is a peptide comprising SEQ ID NO: 2 or a fragment or variant thereof and  $n \geq 2$ .

SEQ ID NO: 2

GIINTLQKYYXRVGRGXAVLSXLPKEEQIGKXSTRGRKXXRRZZ

X comprises any amino acid; Z comprises any amino acid or may be absent. For example, Z may include but is not limited to lysine, ornithine or arginine. In particular, Z may be lysine.

According to another aspect, the present invention relates to an isolated multimer comprising at least two units of a peptide U, wherein the peptide U comprises SEQ ID NO: 2 or a fragment or variant thereof.

The peptide U is repeated in the multimer. In particular, the repeating peptides U are linked together covalently. Further, the peptides U may be linked together through at least one amino acid B. Each B may be an amino acid having at least two amine groups. For example, each B may include but is not limited to lysine, ornithine or arginine.

The repeating unit or the peptide U in any multimer according to the present invention may be any peptide described in WO 2007/126392.

The peptide U may include peptide fragments derived from SEQ ID NO: 2. The fragments may be fragments of any length derived from SEQ ID NO: 2. In particular, the peptide U may comprise SEQ ID NO: 3 or a fragment or variant thereof.

SEQ ID NO: 3

GIINTLQKYYXRVGRGXAVLSXLPKEEQIGKXSTRGRKXXRR

X comprises any amino acid.

The peptide U of the multimer may have a charge of +1 to +11.

According to another aspect, the invention relates to an isolated multimer of formula  $(U)_nB_mZ_j$ , wherein U comprises SEQ ID NO: 3 or a fragment or variant thereof, each B comprises at least one amino acid residue comprising at least two amine groups, Z comprises any amino acid,  $n \geq 2$ ,  $m \geq 1$  and  $j \geq 0$ . B may comprise an amino acid having at least two amine groups. In particular, each B includes but is not limited to lysine, ornithine or arginine.

According to one aspect of the invention, the B and Z in  $(U)_nB_mZ_j$  may both comprise lysine (K) and the formula may be expressed as  $(U)_nK_mK_j$ . According to a further aspect, m may also equal the number n-1 and j may equal to 1 and the formula of the multimer may be expressed as  $(U)_nK_{n-1}K$  (or  $(UK)_n$ ). The peptide U in  $(U)_nK_mK_j$  or  $(U)_nK_{n-1}K$  may comprise SEQ ID NO: 3 or a fragment or variant thereof.

The multimer(s) of the invention may be linear or branched. If the multimer  $(U)_n$  is linear, the peptide U

repeated  $n$  times may comprise SEQ ID NO: 2 or any fragment or variant of SEQ ID NO: 2.

For a branched multimer, for example the multimer  $(U)_n B_m Z_r$ , the multimer may be branched at the terminal  $B_m Z_r$  residues.

The multimers according to the invention may comprise any number of repeating units of the peptide  $U$ . For example, the multimer may comprise 2 to 10, 2 to 20, 2 to 30 repeating units of the peptide  $U$ . Further, the multimer may be a dimer, trimer, tetramer, pentamer, hexamer, heptamer, octamer, nonamer and decamer. In particular,  $n$  may be an even number. More in particular,  $n$  may comprise any number starting from 2 and increasing in multiples of 2.

Further, any amino acid residue of the multimer may comprise an amino acid having at least one protecting group. The protecting groups may comprise Boc, But, Fmoc, Pbf or any other protecting group.

The invention includes a composition or a combination comprising at least one multimer according to any aspect of the invention and at least one other active pharmaceutical ingredient.

The invention also relates to a method of preparing at least one multimer of the invention.

Accordingly, the invention relates to a method of preparing at least one multimer of formula  $(U)_n$ , wherein  $U$  is a peptide comprising SEQ ID NO: 2 or a fragment or variant thereof, the method comprising linking at least two units of  $U$  together.

The multimer(s) of the invention may also be produced by recombinant DNA technology. Any part or the whole of the multimer(s) of the invention may be produced by recombinant DNA technology. For example, the peptide monomers may be synthesised separately by recombinant DNA methods and then linked together to form multimers by chemical methods.

Accordingly, the present invention also relates to an isolated nucleic acid molecule encoding any part of or the whole of the multimer(s) of the invention. The nucleic acid molecule(s) may be inserted into a vector. Further, either the nucleic acid molecule(s) or the vector comprising the nucleic acid molecule(s) may be introduced into a host cell for expressing any part of or the whole of the multimer(s) of the invention.

The multimer(s) of the invention may also be produced de novo by chemical synthesis. For example, the multimer(s) of the invention may be produced by a solid phase peptide synthesis (SPPS) method of the invention.

The invention also relates to a method of preparing at least one multimer of formula:  $(U)_n B_m Z_r$ , wherein  $U$  comprises SEQ ID NO: 3 or a fragment or variant thereof, each  $B$  comprises an amino acid comprising at least two amine groups,  $Z$  comprises any amino acid and  $n \geq 2$ ,  $m \geq 1$  and  $j \geq 0$ .

- (i) providing at least one solid phase;
- (ii) coupling at least a first amino acid  $Z$  to the solid phase;
- (iii) linking at least one protected amino acid residue  $B$  to the coupled first amino acid residue;
- (iv) removing the protecting group(s) from the linked  $B$  residue(s);
- (v) providing additional chain extension by linking protected amino acid residues, according to the sequence of the peptide  $U$  in order from the C-terminus to the N-terminus, wherein after each linking, the protecting groups are removed for the next linking; and
- (vi) terminating the linking of amino acid residues depending on the number of residues to be added.

The protected amino acid  $B$  comprises at least two side chains protected by protecting groups. In particular, each  $B$

includes but is not limited to lysine, ornithine or arginine. The amino acid  $Z$  first coupled to the solid phase may also be but is not limited to lysine, ornithine or arginine.

The method may be extended to produce further multimers. For example, the extended method to produce further multimers further comprises, after step (iv)

- (iv)(a) linking further protected  $B$  residues to the linked  $B$  residue(s);
- (iv)(b) removing the protecting group(s) from the  $B$  residues from (iv)(a);
- (iv)(c) repeating step (iv)(a) and (iv)(b), or
- (iv)(d) proceeding to step (v) and (vi).

The multimers formed depend on the number of amine groups in  $B$ . If the number of amine groups in  $B$  is two, for example in lysine (K) or ornithine, the extended method may produce multimers increasing in multiples of two from the previous multimer. The multimers formed from the extended method will be four, eight, sixteen, thirty-two, sixty-four and so on.

If  $B$  is arginine (R) which has three amine groups (two primary amine groups and one secondary amine group), the first multimer formed will be a trimer. The subsequent multimers formed using arginine will increase in multiples of three.

For each round of multimerisation, combinations of lysine or ornithine with arginine or vice versa may be used for  $B$ , and multimers with different number of repeating units may be produced.

Following completion of the synthesis of the multimer, the multimer may be released from the solid phase.

#### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates the structures of the protected lysine residue Lys(Fmoc), the V2 monomer and the V2 dimer. The lysine (K) residue in bold shows the position which Lys (Fmoc) were incorporated during the synthesis.

FIG. 2 illustrates the synthesis of the V2 dimer by solid-phase peptide synthesis (SPPS).

Step (i) illustrates the removal of protecting group using 20% piperidine in DMF (N,N-Dimethylformamide).

Step (ii) illustrates the coupling reactions with 0.5 M HBTU (N-[1H-benzotriazol-1-yl]-(dimethylamino)methylen-N-methylmethanaminium), 0.5 M HOBT (N-hydroxybenzotriazole), 2 M DIEA (Diisopropylethylamine) in NMP (N-methylpyrrolidone).

Fmoc(Lys) is coupled to the lysine residue bound to the resin. Step (i) is repeated. Two arginine residues (R) are then added to each chain.

Step (iii) illustrates chain extension comprising six cycles of SPPS with 0.5 M HBTU/0.5 M HOBT/2 M DIEA to incorporate the remaining amino acids of the V2 dimer.

Step (iv) illustrates the cleavage reaction to release the dimer from the resin using the cleavage agent 90% TFA (trifluoroacetic acid), 5.0% phenol, 1.5% water, 1.0% TIS (triisopropyl silane), 2.5% EDT (ethane dithiol).

RGRKVVRR (SEQ ID NO: 44) is the repeated in the dimer.

FIG. 3 illustrates that the SPPS method can be used to form multimers increasing in multiples of 2. For example, dimers, tetramers, octamers and further multimers wherein the number of monomer units increases in multiples of two may be produced. The lysine (K) residue in bold italics shows the position which Lys(Fmoc) were incorporated during the synthesis.

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The sequences in FIG. 3 are:

RGRKVVRRKK (SEQ ID NO: 45)  
RGRKVVRR (SEQ ID NO: 46)

FIG. 4 is a graph which illustrates that the V2 dimer does not show cytotoxicity toward human conjunctival epithelial cells in comparison with native hBD3. The results are from four independent experiments and data points show the mean and standard deviations. The X axis indicates the peptide concentration in  $\mu\text{g/ml}$  and the Y axis indicates the % cell viability.

FIG. 5 illustrates the haemolytic effects of wt hBD3 and its C-terminus peptides on rabbit erythrocytes.

FIG. 6 illustrates the cytotoxicity effects of wt hBFD3 and its C-terminus peptides on human conjunctival epithelial cells.

FIG. 7 illustrates an exemplification of a method of preparing a heterodimer.

The sequences in FIG. 7 are:

RGRKVVRR (SEQ ID NO: 44)  
RGRKVVRRVV (SEQ ID NO: 46)

FIG. 8 illustrates an exemplification of a method of preparing a heterotetramer.

The sequences in FIG. 8 are:

RRVVKRGRK (SEQ ID NO: 58)  
RGRKVVRR (SEQ ID NO: 44)  
RGRKVVRRVV (SEQ ID NO: 46)

FIG. 9 illustrates an exemplification of a method of preparing a homogeneous tetramer.

FIG. 10 shows the LPS, Lipid A and  $\text{Mg}^{2+}$  studies. The interaction of B2088 and B2088\_99 with (A) LPS and (B) Lipid A is shown. (C) Competitive inhibition assay showing the effect of exogenous addition of LPS on the inhibitory activity of peptides. (D) Effect of  $\text{Mg}^{2+}$  ion on the MIC of B2088 and B2088\_99.

FIG. 11 shows the bactericidal properties of B2088 and B2088\_99 against (A) *Pseudomonas aeruginosa* ATCC 9027 (Pa 9027) and (B) *P. aeruginosa* ATCC 27853 strains (Pa 27853).

FIG. 12 shows the time kill kinetics of B2088 and B2088\_99 against *P. aeruginosa*.

FIG. 13 shows the outer membrane permeability of B2088 and B2088\_99.

FIG. 14 shows the results of the animal model study with (A) gatifloxacin alone and together with B2088 and (B) gatifloxacin alone and together with B2088\_99.

## DEFINITIONS

Where X is found in a peptide sequence or formula, X represents any amino acid, including protected cysteine residues including but not limited to C(Acm), C(But), C(Cam), C(t-Buthio), C(Bzl), C(4-MeO-Bzl) and C(Mmt).

Variant of a peptide or multimer refers to variations in a peptide sequence (of the multimer) wherein one or more amino acids may be substituted with other amino acids. The substitution is usually conservative, such as with amino acids that has similar properties. The variant(s) generally maintain

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a net charge of +1 to +11. The variant(s) are generally active and have good antimicrobial properties and low cytotoxicity.

A protected amino acid is an amino acid with one or more of its reactive groups modified with an inert molecule, to reduce and/or prevent chemical reactions of the reactive group.

## DETAILED DESCRIPTION OF THE INVENTION

According to a first aspect, the present invention relates to an isolated multimer of defensin peptides of formula  $(U)_n$ , wherein U is a peptide comprising SEQ ID NO: 2 or a fragment or variant thereof and  $n \geq 2$ .

SEQ ID NO: 2  
GIINTLQKYYXRVGRGXAVLSXLPKEEQIGKXSTRGRKXXRRZZ

X may comprise any amino acid; Z comprises any amino acid or may be absent. For example, Z may include but is not limited to lysine, ornithine or arginine. In particular, Z may be lysine.

According to another aspect, the present invention provides an isolated multimer comprising at least two units of a peptide U, wherein the peptide U comprises SEQ ID NO: 2 or a fragment or variant thereof.

The peptide U is repeated in the multimer. In particular, the repeating peptides U are linked together covalently. The peptides U may be linked together through at least one amino acid B. B may comprise an amino acid having at least two amine groups. For example, B may include but is not limited to lysine, ornithine or arginine.

The repeating unit or the peptide U in any multimer according to the present invention may be any peptide described in WO 2007/126392.

The peptide U may include peptide fragments derived from SEQ ID NO: 2. The fragments may be fragments of any length derived from SEQ ID NO: 2. In particular, the peptide U may comprise SEQ ID NO: 3 or fragment or variant thereof.

SEQ ID NO: 3  
GIINTLQKYYXRVGRGXAVLSXLPKEEQIGKXSTRGRKXXRR

X comprises any amino acid.

The peptide U of the multimer may have a charge of +1 to +11.

Further, the peptide U may further comprise any one of SEQ ID NOs: 4 to 58 or fragment or variant thereof.

(38 aa peptide derived from hBD3 C terminus)  
SEQ ID NO: 4  
KYYXRVGRGXAVLSXLPKEEQIGKXSTRGRKXXRRZZ

X comprises any amino acid; Z comprises any amino acid or may be absent.

(36 aa peptide derived from hBD3 C terminus)  
SEQ ID NO: 5  
YXRVGRGXAVLSXLPKEEQIGKXSTRGRKXXRRZZ

X comprises any amino acid; Z comprises any amino acid or may be absent.

(40 aa peptide derived from hBD3 C terminus)  
SEQ ID NO: 6  
LQKYYXRVGRGXAVLSXLPKEEQIGKXSTRGRKXXRRZZ

X comprises any amino acid; Z comprises any amino acid or may be absent.



(29 aa peptide derived from hBD3 C terminus) SEQ ID NO: 7 RXAVLSXLPKEEQIGKXSTRGRKXXRRZZ	(full length hBD3 derived peptide, C replaced with C (Acm) SEQ ID NO: 17
X comprises any amino acid; Z comprises any amino acid or may be absent.	5 GIINTLQKYYC (Acm) RVRGGRC (Acm) C (Acm) VLSALPKEEQIGK
(20 aa peptide derived from hBD3 C terminus) SEQ ID NO: 8 10 KEEQIGKXSTRGRKXXRRZZ	C (Acm) STRGRKC (Acm) C (Acm) RRZZ Z comprises any amino acid or may be absent.
X comprises any amino acid; Z comprises any amino acid or may be absent.	(full length hBD3 derived peptide, C replaced with C (But) SEQ ID NO: 18
(14 aa peptide derived from hBD3 C terminus) SEQ ID NO: 9 KXSTRGRKXXRRZZ	15 GIINTLQKYYC (But) RVRGGRC (But) C (But) VLSALPKEEQIGK C (But) STRGRKC (But) C (But) RRZZ
X comprises any amino acid; Z comprises any amino acid or may be absent.	Z comprises any amino acid or may be absent.
(19 aa peptide derived from hBD3 aa 8-26) SEQ ID NO: 10 KYYXRVRGGRXAVLSXLPK	(full length hBD3 derived peptide, C replaced with C (t-Buthio) SEQ ID NO: 19
X comprises any amino acid	25 GIINTLQKYYC (t-Buthio) RVRGGRC (t-Buthio) C (t-Buthio) V LSALPKEEQIGKC (t-Buthio) STRGRKC (t-Buthio) C (t-Buthio) RRZZ
X comprises any amino acid	Z comprises any amino acid or may be absent
(17 aa peptide derived from hBD3 N-terminus) SEQ ID NO: 11 GIINTLQKYYXRVRGGR	(full length hBD3 derived peptide, C replaced with C (Bzl) SEQ ID NO: 20
X comprises any amino acid	35 GIINTLQKYYC (Bzl) RVRGGRC (Bzl) C (Bzl) VLSALPKEEQIGK C (Bzl) STRGRKC (Bzl) C (Bzl) RRZZ
(full length hBD3 derived peptide, C replaced with W) SEQ ID NO: 12 GIINTLQKYYRVRGGRWAVLSWLPKEEQIGKWSTRGRKWRRZZ	Z comprises any amino acid or may be absent
Z comprises any amino acid or may be absent.	40 (full length hBD3 derived peptide, C replaced with C (4-MeBzl) SEQ ID NO: 21
(full length hBD3 derived peptide, C replaced F) SEQ ID NO: 13 GIINTLQKYYRVRGGRFAVLSFLPKEEQIGKFSTRGRKFFRRZZ	45 GIINTLQKYYC (4-MeBzl) RVRGGRC (4-MeBzl) C (4-MeBzl) VLSALPKEEQIGKC (4-MeBzl) STRGRKC (4-MeBzl) C (4-MeBzl) RRZZ
Z comprises any amino acid or may be absent.	Z comprises any amino acid or may be absent.
(full length hBD3 derived peptide, C replaced Y) SEQ ID NO: 14 50 GIINTLQKYYRVRGGRYAVLSYLPKEEQIGKYSTRGRKYRRZZ	(full length hBD3 derived peptide, C replaced with C (4-MeOBzl) SEQ ID NO: 22
Z comprises any amino acid or may be absent.	55 GIINTLQKYYC (4-MeOBzl) RVRGGRC (4-MeOBzl) C (4-MeOBzl) VLSALPKEEQIGKC (4-MeOBzl) STRGRKC (4-MeOBzl) C (4-MeOBzl) RRZZ
(full length hBD3 derived peptide, C replaced with S) SEQ ID NO: 15 GIINTLQKYYRVRGGRSAVLSLSPKEEQIGKSTRGRKSSRRZZ	Z comprises any amino acid or may be absent.
Z comprises any amino acid or may be absent.	(full length hBD3 derived peptide, C replaced with C (Mmt) SEQ ID NO: 23
(full length hBD3 derived peptide, C replaced with A) SEQ ID NO: 16 GIINTLQKYYRVRGGRAVLSALPKEEQIGKASTRGRKAARRZZ	65 GIINTLQKYYC (Mmt) RVRGGRC (Mmt) C (Mmt) VLSALPKEEQIGK C (Mmt) STRGRKC (Mmt) C (Mmt) RRZZ
Z comprises any amino acid or may be absent.	Z comprises any amino acid or may be absent.

(full length hBD3 derived peptide, C replaced with modified C

SEQ ID NO: 24  
GIINTLQKYYXRVRGGRXXVLSALPKKEEQIGKXSTRGRKXXRRZZ

X comprises C(Acm), C(But), C(t-Buthio), C(Bzl), C(4-MeBzl), C(4-MeOBzl) or C(Mmt); Z comprises any amino acid or may be absent

(hBD3 derived 10 aa C-terminus fragment, C replaced with any amino acid X)

SEQ ID NO: 25  
RGRKXXRRZZ

X comprises any amino acid; Z comprises any amino acid or may be absent.

(hBD3 derived 10 aa C-terminus fragment, C replaced with W)

SEQ ID NO: 26  
RGRKWWRRZZ

Z comprises any amino acid or may be absent.

(hBD3 derived 10 aa C-terminus fragment, C replaced with F)

SEQ ID NO: 27  
RGRKFFRRZZ

Z comprises any amino acid or may be absent.

(hBD3 derived 10 aa C-terminus fragment, C replaced with Y)

SEQ ID NO: 28  
RGRKYRRZZ

Z comprises any amino acid or may be absent.

(hBD3 derived 10 aa C-terminus fragment, C replaced with L)

SEQ ID NO: 29  
RGRKLLRRZZ

Z comprises any amino acid or may be absent.

(hBD3 derived 10 aa C-terminus fragment, C replaced with I)

SEQ ID NO: 30  
RGRKIIRZZ

Z comprises any amino acid or may be absent.

(hBD3 derived 10 aa C-terminus fragment, C replaced with H)

SEQ ID NO: 31  
RGRKHRRZZ

Z comprises any amino acid or may be absent.

(hBD3 derived 10 aa C-terminus fragment, C replaced with C(Acm), C(But), C(t-Buthio), C(Bzl), C(4-MeBzl), C(4-MeOBzl) or C(Mmt)...

SEQ ID NO: 32  
RGRKXXRRZZ

X comprises C(Acm), C(But), C(t-Buthio), C(Bzl), C(4-MeBzl), C(4-MeOBzl) or C(Mmt); Z comprises any amino acid or may be absent.

(hBD3 derived 10 aa C-terminus fragment, C replaced with V)

RGRKVVRZZ

Z comprises any amino acid or may be absent.

(hBD3 derived 10 aa C terminus peptide)

SEQ ID NO: 34  
RGRKCCRRZZ

Z comprises any amino acid or may be absent.

(hBD3 derived 10 aa C-terminus peptide, C replaced with C(Acm), C(But), C(t-Buthio), C(Bzl), C(4-MeBzl), C(4-MeOBzl) or C(Mmt).

SEQ ID NO: 35  
RGRKXXRRKK

X comprises C(Acm), C(But), C(t-Buthio), C(Bzl), C(4-MeBzl), C(4-MeOBzl) or C(Mmt).

(hBD3 derived C-terminus peptide excluding terminal KK, C replaced with any amino acid)

SEQ ID NO: 36  
RGRKXXRR

(hBD3 derived C-terminus peptide excluding terminal KK, C replaced with W, W2-8AA)

SEQ ID NO: 37  
RGRKWWRR

(hBD3 derived C-terminus peptide excluding terminal KK, C replaced with F)

SEQ ID NO: 38  
RGRKFFRR

(hBD3 derived C-terminus peptide excluding terminal KK, C replaced with Y, Y2-8AA)

SEQ ID NO: 39  
RGRKYRR

(hBD3 derived C-terminus peptide excluding terminal KK, C replaced with L)

SEQ ID NO: 40  
RGRKLLRR

(hBD3 derived C-terminus peptide excluding terminal KK, C replaced with I)

SEQ ID NO: 41  
RGRKIIR

(hBD3 derived C-terminus peptide excluding terminal KK, C replaced with H)

SEQ ID NO: 42  
RGRKHRR

(hBD3 derived C-terminus peptide excluding terminal KK, C replaced with C(Acm), C(But), C(t-Buthio), C(Bzl), C(4-MeBzl), C(4-MeOBzl) or C(Mmt).

SEQ ID NO: 43  
RGRKXXRR

X comprises C(Acm), C(But), C(t-Buthio), C(Bzl), C(4-MeBzl), C(4-MeOBzl) or C(Mmt).

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(hBD3 derived C-terminus peptide excluding terminal KK, C replaced with V, V2-8AA)	SEQ ID NO: 44
RGRKVVRR	
(V2 monomer)	
RGRKVVRRKK	SEQ ID NO: 45
(V4 monomer, V4-10 AA)	
RGRKVVRRVV	SEQ ID NO: 46
(Y4 monomer, Y4-10 AA)	
RGRKYVRRYY	SEQ ID NO: 47
(W4 monomer, W4-10 AA)	
RGRKWVRRWW	SEQ ID NO: 48
(V3 monomer)	
RVRKVVRR	SEQ ID NO: 49
(V2R monomer)	
RRRKVVRR	SEQ ID NO: 50
(V2D monomer)	
RDRKVVRR	SEQ ID NO: 51
(E2 monomer)	
RGRKEERR	SEQ ID NO: 52
(K2 monomer)	
RGRKKKRR	SEQ ID NO: 53
RRRRRRRRRR	SEQ ID NO: 54
VVVV	SEQ ID NO: 55
YYYY	SEQ ID NO: 56
RRVVKRGR	SEQ ID NO: 57
RRVVKRGRK	SEQ ID NO: 58
RGRKGGRR	SEQ ID NO: 59

According to another aspect, the invention relates to an isolated multimer of formula  $(U)_n B_m Z_j$ , wherein U comprises SEQ ID NO: 3 or a fragment or variant thereof, B comprises an amino acid having at least two amine groups, Z comprises any amino acid,  $n \geq 2$ ,  $m \geq 1$  and  $j \geq 0$ . In particular, each B may include but is not limited to lysine, ornithine or arginine.

According to one aspect of the invention, the B and Z in  $(U)_n B_m Z_j$  may both comprise lysine (K) and the formula may be expressed as  $(U)_n K_m K_j$ . According to a further aspect, m may also equal the number n-1 and j may equal to 1 and the formula of the multimer may be expressed as  $(U)_n K_{n-1} K$  (or  $(UK)_n$ ). The peptide U in  $(U)_n K_m K_j$  or  $(U)_n K_{n-1} K$  may comprise SEQ ID NO: 3 or a fragment or variant thereof.

The peptide U in the formula  $(U)_n B_m Z_j$ ,  $(U)_n K_m K_j$  or  $(U)_n K_{n-1} K$  may comprise SEQ ID NO: 3 or a fragment or variant thereof, such as RGRKXXRR (SEQ ID NO: 36) or any one of SEQ ID NOs: 37-44.

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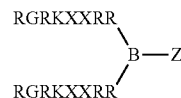
For example, if  $m=n-1$ ,  $j=1$ ,  $B=Z=K$ , U comprises SEQ ID NO: 44, the multimer has the formula (SEQ ID NO: 44)<sub>n</sub> K<sub>n-1</sub>K. If  $n=2$ , the multimer has the formula (SEQ ID NO: 44)<sub>2</sub>KK.

The multimer(s) of the invention may be linear or branched. If the multimer  $(U)_n$  is linear, the peptide U repeated n times may comprise SEQ ID NO: 2 or any fragment or variant of SEQ ID NO: 2, such as any one of SEQ ID NOs: 3-58. For example, the multimer may comprise (SEQ ID NO: 35)<sub>n</sub>, (SEQ ID NO: 36)<sub>n</sub> or (SEQ ID NO: 45)<sub>n</sub>.

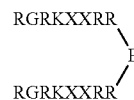
For a branched multimer, for example the multimer  $(U)_n B_m Z_j$ , the multimer may be branched at the terminal B<sub>m</sub>Z<sub>j</sub> residues.

The multimer may comprise any number of repeating units. For example, the multimer may comprise 2 to 10, 2 to 20, 2 to 30 repeating subunits. In addition, the multimer may be a dimer, trimer, tetramer, pentamer, hexamer, heptamer, octamer, nonamer and decamer.

For example, for a branched dimer of formula (SEQ ID NO: 36)<sub>2</sub>BZ, the branched dimer may comprise the structure:



As an alternative, Z may be absent and the dimer may have the structure:



In particular, for any of the multimer according to the invention, X may be V. The V2 monomer has the sequence RGRKVVRR (SEQ ID NO: 44).

The invention includes a composition or a combination comprising at least one isolated multimer according to any aspect of the invention and at least one other active pharmaceutical ingredient.

The composition or combination may comprise at least one multimer comprising the formula  $(U)_2 KK$ , wherein U comprises SEQ ID NO: 2 or a fragment or variant thereof; and at least one other active pharmaceutical ingredient. U may be any one of SEQ ID NOs: 3-59.

For example, the composition or combination comprises the multimer of formula (SEQ ID NO: 36)<sub>n</sub> K<sub>n-1</sub>K and at least one other active pharmaceutical ingredient.

In particular, the composition or combination comprises the multimer of formula (SEQ ID NO: 44)<sub>n</sub> K<sub>n-1</sub>K and at least one other active pharmaceutical ingredient.

More in particular, the composition or combination comprises the multimer of formula (SEQ ID NO: 44)<sub>2</sub>KK and at least one other active pharmaceutical ingredient.

The active pharmaceutical ingredient may comprise an antimicrobial compound. The multimer and the antimicrobial compound act synergistically. Any suitable antimicrobial compound may be used. Examples of antimicrobial compounds include but are not limited to chloramphenicol, penicillins, macrolides, fluoroquinolones, aminoglycosides,  $\beta$ -lactams and quinolones.

An example of a penicillin is carbenicillin.

An example of a macrolide is erythromycin.

An example of a fluoroquinolone is gatifloxacin.

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Examples of aminoglycosides include but are not limited to gentamycin, kanamycin and streptomycin.

An example of a  $\beta$ -lactam is imipenam.

An example of a quinolone is nalidixic acid.

The composition or combination may comprise the multimer (SEQ ID NO: 44)<sub>2</sub>KK and at least one antimicrobial compound selected from the group consisting of chloramphenicol, penicillins, macrolides, fluoroquinolones, aminoglycosides,  $\beta$ -lactams or quinaolones.

In a further example, the composition or combination comprises the multimer (SEQ ID NO: 44)<sub>2</sub>KK and at least one antimicrobial compound selected from the group consisting of carenicillin, chloramphenicol, erythromycin, gatifloxacin, gentamycin, imipenam, kanamycin, nalidixic acid and streptomycin.

In one specific example, the composition or combination comprises (SEQ ID NO: 44)<sub>2</sub>KK and gatifloxacin.

The composition comprising at least one isolated multimer according to any aspect of the invention and at least one other active pharmaceutical ingredient may be a pharmaceutical composition, an antimicrobial composition, an eye drop composition and/or solution(s), a contact lens solution(s) or a composition for coating a device.

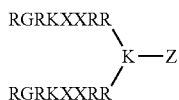
According to a further aspect, the invention also relates to a method of preparing at least one multimer of formula: (U)<sub>n</sub>K<sub>m</sub>Z<sub>j</sub>, wherein U comprises SEQ ID NO: 3 or a fragment or variant thereof, Z comprises any amino acid and  $n \geq 2$ ,  $m \geq 1$  and  $j = 1$ .

- (i) providing at least one solid phase;
- (ii) coupling at least a first amino acid residue Z to the solid phase;
- (iii) linking at least one protected K residue to the coupled first amino acid residue;
- (iv) removing the protecting group(s) from the linked K residue(s);
- (v) providing additional chain extension by linking protected amino acid residues, according to the sequence of the peptide U in order from the C-terminus to the N-terminus, wherein after each linking, the protecting groups are removed for the next linking; and
- (vi) terminating the linking of amino acid residues depending on the number of residues to be added.

The amino acid Z first coupled to the solid phase may be any amino acid. For example, Z may include but is not limited to lysine, ornithine or arginine.

Following completion of the synthesis of the multimer, the multimer may be released from the solid phase.

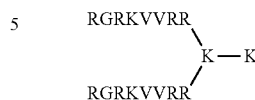
For example, performing the above steps for the peptide sequence RGRKXXRR (SEQ ID NO: 36) will produce the following dimer. The lysine (K) residue in bold italics shows the positions which Lys(Fmoc) was incorporated during the synthesis.



In the synthesis method, the Z and lysine residues are shared between the monomer units.

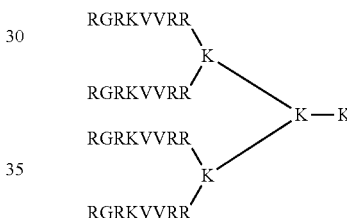
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In particular, when X is V and Z is K, the dimer below is produced. This dimer is the V2 dimer, (SEQ ID NO: 44)<sub>2</sub>KK.



- The method may be extended to produce further multimers. For example, the extended method to produce further multimers further comprises, after step (iv)
  - (iv)(a) linking further protected lysine residues to the linked second lysine residue(s);
  - (iv)(b) removing the protecting group(s) from the lysine residues from (iv)(a);
  - (iv)(c) repeating step (iv)(a) and (iv)(b), or
  - (iv)(d) proceeding to step (v) and (vi).

This extended method will produce multimers increasing in multiples of two from the previous multimer. The multimers formed from the extended method will be four, eight, sixteen, thirty-two, sixty-four and so on. Repeating the additional steps of the extended method once produces a tetramer, as shown below. If X is V and B and Z are K, then the tetramer is known as the V2-tetramer. The repeated unit in the V2 tetramer is RGRKVRR (SEQ ID NO: 44).



Further, repeating the additional steps of the extended method twice will produce an octamer. The multimer(s) of the invention may exclude the terminal K residue.

The multimers prepared as described above are homogeneous multimers (homo-multimers), such that a peptide monomeric unit is repeated in the multimers. The peptide multimers of the invention may be homo-dimers, homo-tetramers or other homo-multimers.

Further, the method of preparation may be extended to prepare heterogeneous multimers, such that the peptide units in the multimers are different. When preparing the homo-multimers, the protected amino acid B have at least two side chains protected by the same protecting group. However, for preparing a heterogeneous multimer, for example, a heterogeneous dimer, a differentially protected amino acid C may be used, wherein the side groups available for chain extension are protected by at least two different protection groups. In this case, a first protection group may be removed to allow peptide chain extension from at least a first reactive side group. After the first peptide chain extension(s) are completed, the other protection group may be removed to allow subsequent chain extension from at least a second reactive side group. In this way, the first peptide chain and the second peptide chain may have different amino acid sequences.

According to another aspect, the invention relates to a method of preparing a peptide multimer of formula [(U<sup>1</sup>)(U<sup>2</sup>)]<sub>n/2</sub>(C)<sub>n/2</sub>B<sub>m</sub>Z wherein each of U<sup>1</sup> and U<sup>2</sup> comprises a peptide with U<sup>1</sup> ≠ U<sup>2</sup>,  $n = 2^x$ , where  $x = 0$  or a positive integer,  $m = 1$  or 0; comprising the steps of:

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- (i) providing at least one solid phase;
- (ii) coupling at least a first amino acid Z to the solid phase;
- (iii) optionally linking at least one amino acid B to the coupled Z;
- (iii) linking at least one protected amino acid C to Z or B; wherein C comprises at least two differentially protected groups;
- (iv) removing a first protecting group from the linked amino acid C to expose a first reactive side chain;
- (v) providing chain extension of a first peptide U<sup>1</sup> to the first reactive side chain of C;
- (vi) removing a second protecting group from the linked B amino acid to expose at least a second reactive side chain; and
- (viii) providing chain extension of a second peptide U<sup>2</sup> to the second reactive side chain of C.

A heterogeneous peptide multimer may thus be prepared.

If the optional step (ii) is omitted, a hetero-dimer is formed. A hetero-tetramer may be prepared if the optional step (ii) is performed once. Other heterogeneous multimers may be prepared by repeating step (ii) accordingly.

According to another aspect, the invention relates to an isolated peptide multimer of formula  $[(U^1)(U^2)]_{n/2}(C)_{m/2}B_mZ$ , wherein U<sup>1</sup> and U<sup>2</sup> comprises a peptide sequence with U<sup>1</sup>≠U<sup>2</sup>, C and B each comprises an amino acid with at least two amine groups, Z comprises any amino acid, n=2<sup>x</sup>, where x=0 or a positive integer, m=1 or 0.

Each of C, B and Z may include but is not limited to lysine (K), ornithine or arginine (R).

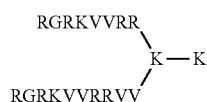
In particular, U<sup>1</sup> or U<sup>2</sup> may each be a peptide comprising SEQ ID NO: 2 or a fragment or variant thereof.

U<sup>1</sup> may comprise but is not limited to any one of SEQ ID NOs: 1 to 58.

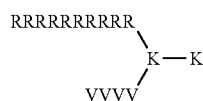
U<sup>2</sup> may comprise but is not limited any one of SEQ ID NOs: 1 to 58.

If n=2 and m=1, the multimer is a heterogenous dimer of formula  $[(U^1)(U^2)]CZ$ .

For example, U<sup>1</sup> may comprise RGRKVVRR (SEQ ID NO: 44) and U<sup>2</sup> may comprise RGRKVVRRVV (SEQ ID NO: 46), C and Z are K, m=0, n=2. The structure of the heterogeneous dimer comprises:

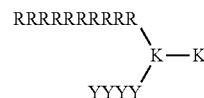


For example U<sup>1</sup> may comprise RRRRRRRRRR (SEQ ID NO: 54) and U<sup>2</sup> may comprise VVVV (SEQ ID NO: 55), C and Z are K, m=0, n=2. The structure of the heterogeneous dimer comprises:



For example U<sup>1</sup> may comprise RRRRRRRRRR (SEQ ID NO: 54) and U<sup>2</sup> may comprise YYYYY (SEQ ID NO: 56), C and Z are K, m=0, n=2. The structure of the heterogeneous dimer comprises:

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If n=4 and m=1, the heterogenous multimer is a heterogeneous tetramer. A heteromer may be of formula  $[(U^1)(U^2)]_2(C)_2B_1Z$ .

The method of the invention may also be extended to synthesise other multimeric peptides, such as trimers. Both homo-trimers and hetero-trimers may be synthesised.

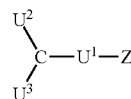
A method of preparing a peptide trimer of formula U<sup>3</sup>U<sup>2</sup>CU<sup>1</sup>Z, wherein U<sup>1</sup>, U<sup>2</sup> and U<sup>3</sup> each comprises a peptide, C comprises an amino acid comprising at least two amine groups and Z comprises any amino acid, the method comprising the steps of:

- (i) providing at least one solid phase;
- (ii) coupling at least a first amino acid Z to the solid phase; and
- (iii) providing chain extension of peptide U<sup>1</sup> to Z; wherein, (A) the peptide trimer is a heterogeneous peptide of formula U<sup>3</sup>U<sup>2</sup>(C)U<sup>1</sup>Z, the method further comprises:
  - (iv) linking a differentially protected amino acid C to an amino acid of peptide U<sup>1</sup>;
  - (v) removing a first protecting group from the linked amino acid C;
  - (vi) providing for chain extension of peptide U<sup>2</sup> to amino acid C;
  - (vii) removing a second protecting group from the linked amino acid C; and
  - (viii) providing for chain extension of peptide U<sup>3</sup> to amino acid C;
- (B) U<sup>2</sup>=U<sup>3</sup>=U<sup>1</sup>, C=B, and the peptide trimer comprises formula (U<sup>1</sup>)<sub>2</sub>BU<sup>1</sup>Z, the method further comprises:
  - (iv) linking a protected amino acid B to an amino acid of peptide U<sup>1</sup>;
  - (v) removing the protecting groups from the linked amino acid B; and
  - (vi) providing for chain extension of two peptides U<sup>1</sup> to the amino acid B; or
  - (C) U<sup>2</sup>=U<sup>3</sup>≠U<sup>1</sup>, C=B, and the peptide comprises formula (U<sup>2</sup>)<sub>2</sub>B(U<sup>1</sup>)Z, the method further comprises:
    - (iv) linking a protected amino acid B to an amino acid of peptide U<sup>1</sup>;
    - (v) removing the protecting groups from the linked amino acid B; and
    - (vi) providing for chain extension of at least two units of peptide U<sup>2</sup> to amino acid B.

In the case of (A) and (C) above, a heterogenous peptide trimer may thus be prepared. In the case of (B) above, a homogeneous peptide trimer may thus be prepared.

According to another aspect, the invention relates to an isolated peptide trimer of formula U<sup>3</sup>U<sup>2</sup>CU<sup>1</sup>Z; wherein U<sup>1</sup>, U<sup>2</sup>, U<sup>3</sup> each comprises a peptide sequence; C comprises an amino acid comprising at least two amine groups and Z comprises any amino acid. In particular, the peptide is branched at amino acid C.

The structure of the isolated peptide trimer comprises:



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Each of C, B and Z may include but is not limited to lysine (K), ornithine or arginine (R).

In particular,  $U^1$ ,  $U^2$  or  $U^3$  may each comprise SEQ ID NO: 2 or a fragment or variant thereof.

For example,  $U^1$ ,  $U^2$  or  $U^3$  may each comprise any one of SEQ ID NOs: 1 to 58. For a heterogeneous peptide trimer, two of the peptides may comprise the same sequence with the third peptide of a different sequence.

For the heterogeneous peptide trimer,  $U^3U^2CU^1Z$ ,  $U^1$ ,  $U^2$  and  $U^3$  may be but need not be different peptide sequences. For example,  $U^1$  and  $U^2$  may comprise the same sequence while  $U^3$  may comprise a different sequence. Alternatively,  $U^1$  and  $U^3$  may comprise the same sequence with  $U^2$  having the same sequence.

If  $U^2=U^3 \neq U^1$  and  $C=B$ , the peptide is of formula  $(U^2)_2BU^1Z$ .

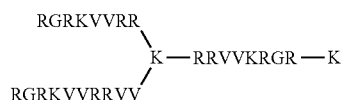
If  $U^1=U^3 \neq U^2$ , the peptide is of formula  $U^1U^2CU^1Z$ .

If  $U^1=U^2 \neq U^3$ , the peptide is of formula  $U^3U^1CU^1Z$ .

However, if  $U^1=U^2=U^3$  and  $C=B$ , the peptide is of formula  $(U^1)_2BU^1Z$  (homogeneous peptide trimer).

An example of a heterogeneous peptide trimer with and its synthesis is illustrated in FIG. 8 and described in Example 1D.

Accordingly, the invention relates to an isolated peptide trimer of formula  $U^3U^2KU^1K$ ; wherein  $U^1$  comprises RRWKRGR (SEQ ID NO: 57) and  $U^2$  comprises RGRKV-VRR (SEQ ID NO: 44) and  $U^3$  comprises RGRKVVRW (SEQ ID NO: 46). This heterogeneous trimer is the V2V2V4-heterotrimer and has the structure:



The multimers of the present invention possess antimicrobial properties. Accordingly, the multimers may be used for inhibiting and/or reducing the growth of microorganisms.

The present invention also provides a method of inhibiting and/or reducing the growth of microorganisms comprising contacting the microorganism with at least one multimer of the invention.

The present invention also provides a method of treating at least one microbial infection comprising administering to a subject at least one multimer of the invention. The invention further provides inhibiting and/or reducing the growth of at least one microorganism in a subject comprising administering to the subject at least one multimer of the invention.

The composition or combination comprising at least one isolated multimer according to the any aspect of the invention and at least one other active pharmaceutical ingredient may be used for inhibiting and/or reducing the growth of microorganisms.

The invention further includes a method of inhibiting and/or reducing the growth of microorganisms comprising contacting at least one microorganism with at least one isolated multimer according to any aspect of the invention and at least one other active pharmaceutical ingredient.

Accordingly, the method of inhibiting and/or reducing the growth of microorganisms comprises contacting at least one microorganism with at least one multimer comprising the formula  $(U)_nK_{n-1}K$ , wherein U comprises SEQ ID NO: 2 or a fragment or variant thereof; and at least one other antimicrobial compound.

For example, the method of inhibiting and/or reducing the growth of microorganisms comprises contacting at least one

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microorganism with at least one multimer comprising the formula  $(U)_2KK$ , wherein U comprises SEQ ID NO: 2 or a fragment or variant thereof; and at least one other antimicrobial compound.

The isolated multimer and the antimicrobial compound may be used separately or together.

The invention includes a method of treating at least one microbial infection comprising administering to a subject at least one isolated multimer according to any aspect of the invention and at least one other active pharmaceutical ingredient. The invention further includes inhibiting and/or reducing the growth of at least one microorganism in a subject comprising administering to the subject at least one isolated multimer according to the invention and at least one active pharmaceutical ingredient.

Accordingly, the method of treating at least one microbial infection comprising administering to a subject at least one isolated multimer comprising the formula  $(U)_nK_{n-1}K$ , wherein U comprises SEQ ID NO: 2 or a fragment or variant thereof; and at least one other active pharmaceutical ingredient.

For example, the method of treating at least one microbial infection comprising administering to a subject at least one multimer comprising the formula  $(U)_2KK$ , wherein U comprises SEQ ID NO: 2 or a fragment or variant thereof; and at least one other active pharmaceutical ingredient.

For the method of treating at least one microbial infection and the method of inhibiting and/or reducing the growth of microorganisms; the U in the multimer may be U any of SEQ ID NOs: 3-59 as defined above. In particular, the U in the multimer and the antimicrobial compound may be as defined for the composition or combination.

The isolated multimer and the active pharmaceutical ingredient may be administered separately or together. For example, the isolated multimer and the active pharmaceutical ingredient may be used or administered separately at the same time or one after the other, in any order. Alternatively, the isolated multimer and the active pharmaceutical ingredient may be mixed together in a composition and used or administered together.

The microorganism may be a virus, fungus or bacteria.

Accordingly, the present invention also relates to the use of a multimer according to any aspect of the invention in the manufacture of an antimicrobial composition. The antimicrobial composition may be used for inhibiting and/or reducing the growth of at least one microorganism, for example, in a subject.

The present invention also includes the use of a multimer according to any aspect of the invention in the manufacture of a medicament for treating at least one microbial infection.

Accordingly, the multimer(s) of the invention may be formulated into antimicrobial compositions and/or pharmaceutical compositions. The antimicrobial and/or pharmaceutical compositions may be formulated for topical, oral, parenteral administration or for administration by inhalation. The multimer(s) of the invention may also be formulated in eye drop composition(s) and/or solution(s) and/or contact lens solution(s).

The multimer(s) of the invention may also be formulated into compositions for coating devices. The devices include medical devices such as but not limited to a catheter, a needle, a sheath, a stent or a dressing.

The invention further includes kit(s) comprising at least one multimer according to the invention, at least one antimicrobial composition and/or pharmaceutical composition comprising at least one multimer according to the invention.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

### EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Sambrook and Russel, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (2001).

#### Example 1

##### Method for Solid-Phase Peptide Synthesis (SPPS) of Multimers

###### (A) Synthesis of Homogeneous Dimer

The method for the synthesis of the peptides is adapted from Krajewski et al., (2004). Fluorenylmethoxycarbonyl (Fmoc)-protected L-amino acids and resin were purchased from Advanced Automated Peptide Protein TECHNOLOGIES, AAPPTEC (KY, US) and used with the following side-chain protective groups: Lys(Fmoc) was incorporated only at second residue from C-terminus for synthesis of dimers, Arg(pbf), Lys(Boc), Tyr(But), Trp(Boc) and Fmoc-Lys(Boc)-Wang resin (substitution 0.72 mmol/g). Syntheses of dimers were carried out on Apex 396 (Advanced ChemTech) by Fmoc chemistry.

Commercially available Fmoc-Boc-Lys-Wang resin was used as the starting point. Alternatively, coupling of the first amino acid to the Wang resin was carried out with 0.5 M DIC (N,N'-diisopropylcarbodiimide). Subsequent coupling reactions (or acylation) were carried out with 0.5M HBTU/0.5M HOBT/2M DIEA in NMP. As an alternative, the coupling reactions may also be carried out with HBTU-HOBT in DMF at a synthesis scale of 0.08 mmol. Fmoc deprotection was carried out with 20% piperidine in DMF.

The resins were treated with 90% TFA (trifluoroacetic acid), 5.0% phenol, 1.5% water, 1.0% TIS (trisopropyl silane), 2.5% EDT (ethane diol) to release the multimer from the resins. Alternatively, the resulting peptidyl resins may be treated with a freshly prepared mixture of TFA/TIS/phenol/Thionisole/water (90/1/2.5/5/1.5, the ratio of volume percent) for 2-3 h at room temperature.

The crude peptides were then precipitated by filtration into ice-cold diethyl ether, separated by centrifugation, washed three times with ice-cold ether and dried by automated evaporation of ether and other remaining or residual solvents in crude solid products in fume hood or dried under vacuum at room temperature. The precipitate dried directly from the ether resulted in a TFA (trifluoroacetic acid) salt. TFA salts may affect the pH of peptide solutions or the viability of cells in culture. Lyophilization from 2 ml 2% acetic acid replaces the TFA salt with an acetic salt, which allows easier handling the other peptides and removes trace amounts of scavengers.

The scheme of the synthesis method is illustrated in FIG. 2.

High yields of crude dimer and purified dimer may be obtained from this SPPS method. For example, yields of crude and purified V2-dimer are 90 and 27%, respectively. Further, 50 mg purified V2-dimer may be obtained based on 0.08 mmol synthesis scale.

The method described is an example of the synthesis method but modifications to the method may be made. For

example, any method for the protection and deprotection of the amino acid residues may be used in the SPPS method.

###### (B) Synthesis of Homogeneous Tetramer

The synthesis method may be extended to the synthesis of a homogeneous tetramer as illustrated in FIG. 9.

Following deprotection of the Fmoc-Lys linked to the immobilised  $\text{NH}_2$ -Lys(Boc), two Fmoc-Lys residues were then linked to the Lys. Further amino acids chain extension were then performed to produce a homogeneous tetramer (V2 tetramer) as illustrated. The conditions for the deprotection (step i), coupling (step ii) and chain extension were the same as for the preparation of the homogeneous dimer.

###### (C) Synthesis of Heterogeneous Dimer

For the synthesis of a heterogeneous dimer as illustrated in FIG. 7, the differentially protected amino acid Fmoc-Lys (Aloc)-OH was used as the branched site to prepare a heterodimer. The Fmoc and Aloc groups have different reactivity with Aloc being stable under the basic conditions used to remove Fmoc.

The deprotection of the immobilised Fmoc-NH-Lys(Boc)-Wang resin was performed as described above.

As illustrated in FIG. 7, Fmoc-Lys(Aloc)-OH was then linked to the lysine residue attached to the Wang resin (coupling step ii). Only the Fmoc group of Fmoc-Lys(Aloc) was removed (Fmoc deprotection) and extension of the first chain was performed with (step iii). When all the amino acid residues in the first chain had been linked, (i.e. the first chain completed), the Aloc group on the Lys(Aloc) was removed (Aloc deprotection) using a Palladium catalyst and the second chain extension was performed. When all the amino acid residues in the second chain had been linked (i.e. second chain also completed), the product was released by acid cleavage. Any remaining protecting groups used to protect reactive groups of the amino acid residues as appropriate (eg Boc, pbf, Mtr) during the synthesis were then removed.

###### (D) Synthesis of Heterogeneous Peptide Trimer

An example of the synthesis of a heterogeneous peptide trimer is illustrated in FIG. 8. The first extension to the immobilised lysine (K) residue is a first peptide ( $U^1$ ) of RGRKVVRR (SEQ ID NO: 57). Subsequently, an Fmoc-Lys(Aloc)-OH is linked to the R residue at the N terminus of the peptide RGRKVVRR. The Fmoc group is then removed and a second peptide ( $U^2$ ) of RGRKVVRR (SEQ ID NO: 44) is added by chain extension to the first amine group of the Lys(Aloc) residue. Following the second chain extension, the Aloc group is removed and a third peptide ( $U^3$ ) of RGRKVVRRV (SEQ ID NO: 46) is added by chain extension to the second amine group of the Lys residue.

Alternatively, Fmoc-Lys(Fmoc)-OH may also be added after the extension of the first peptide  $U^1$  is completed. A peptide  $U^2$  may be extended in duplicate to form the heterogeneous trimer  $U^2U^2(K)U^1K$ .

For a homogeneous peptide trimer, Fmoc-Lys(Fmoc)-OH may be used after the extension of the peptide U is completed. The same peptide U may then be extended in duplicate simultaneously to form the homogeneous trimer.

#### Example 2

##### Antimicrobial Assay

The method for testing antimicrobial activity of antimicrobial peptides used an absolute killing procedure as described below.

###### Preparation of Test Organisms

The test organisms were either reference cultures obtained from American Type Culture Collection (ATCC) or clinical

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isolates obtained from the Department of Pathology, Singapore General Hospital. All cultures used in the study were not more than 5 passages from the source.

The bacterial cultures were grown on Trypticase Soy Agar (TSA) slants, and the yeast culture on Sabouraud Dextrose Agar (SDA) slants at 35° C. for 16 hours. The organisms were harvested by centrifugation and washing twice in Phosphate buffer, pH 7.2 (United States Pharmacopeia, USP) at 20° C.

#### Test Organisms Used

The following organisms were used in the study:

1. *Bacillus cereus* ATCC 11778
2. *Candida albicans* ATCC 10231
3. Clinical *Pseudomonas aeruginosa* PAE230 DR4877/07. Source: Sputum
4. Clinical *Pseudomonas aeruginosa* PAE239 DM5790/07. Source: Wound
5. Clinical *Pseudomonas aeruginosa* PAE240 DU14476/07. Source: Urine
6. Clinical *Pseudomonas aeruginosa* PAE249 DM15013. Source: Wound
7. Clinical *Pseudomonas aeruginosa* 07DM023257. Source: Eye
8. Clinical *Pseudomonas aeruginosa* 07DM023376. Source: Eye
9. Clinical *Pseudomonas aeruginosa* 07DM023155. Source: Eye
10. Clinical *Pseudomonas aeruginosa* 07DM023104. Source: Eye
11. *Pseudomonas aeruginosa* ATCC 9027
12. *Pseudomonas aeruginosa* ATCC 27853
13. *Escherichia coli* ATCC25922
14. Clinical *Escherichia coli* DB16027 Source: Blood
15. Clinical *Escherichia coli* DU46381R Source: Urine
16. Methicillin-resistant *Staphylococcus aureus* (MRSA) DM09808R Source: Eye
17. Clinical *Staphylococcus aureus* DM4001 Source: Eye
18. Clinical *Candida albicans* DF2672R Source: Urine
19. *Fusarium solani* ATCC 36031

#### Preparation of Test Solutions of Compounds

The freeze-dried antimicrobial compound was dissolved in purified water and distributed into screw-capped plastic tubes at a concentration of 1,000 micro-grams ( $\mu\text{g}$ ) per ml. These served as stock solutions and were kept at -20° C.

On the day of conducting the test, one tube of the stock solution was defrosted and diluted in purified water to a concentration of 500  $\mu\text{g}/\text{ml}$ . Thereafter, further dilutions were carried out in either USP Phosphate Buffer, pH 7.2 or other solutions (including but not limited to 10 mM sodium phosphate buffer, pH 7.4, 10 mM potassium buffer 7.2 or 155.2 mM NaCl), to the test concentrations required, normally ranging from 6.25  $\mu\text{g}/\text{ml}$  to 50  $\mu\text{g}/\text{ml}$ .

Ten micro-liters ( $\mu\text{l}$ ) of the test organism of standardised concentration was inoculated into one ml of the compound test solution of specific test concentration to provide a final count of between  $1 \times 10^5$  cfu/ml and  $1 \times 10^6$  cfu/ml as far as possible. The inoculated test solutions were then incubated at 35° C. for 4 hours. The incubation temperature and time may be varied as required.

After incubation, the antimicrobial activity of the test solutions was inactivated by a 10-fold dilution in D/E Neutralising Broth (NB). Further dilutions were carried out in the NB, and plated out in TSA for bacteria, and SDA for yeast culture. The plates were incubated at 35° C. for 72 hours. The viable count of the survivor organisms was then determined.

As an inoculum control, the test organism was inoculated, parallel to the Test, into the buffer used in the preparation of the test solutions instead and incubated under the same con-

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dition as the Test. The viable count of the inoculum control was determined as for the Test.

The antimicrobial activity of the compound was expressed as log reduction calculated by subtracting the log number of colony forming units (cfu) of the test organism survivor after 4 hours' exposure time at 35° C. from the log number of cfu of the inoculum control of the test organism.

The method described above was used for Examples 3-7 and in particular, the method for gentamicin in Example 6 is similar except that gentamicin was substituted for the antimicrobial compound.

#### Example 3

##### Antimicrobial Activity of V2 Dimer Against *P. aeruginosa* ATCC 9027

The antimicrobial properties of the V2 dimers were tested on *Pseudomonas aeruginosa* ATCC 9027.

The V2 dimer shows antimicrobial activity against *P. aeruginosa* ATCC 9027 in USP Phosphate buffer (prepared according to US Pharmacopeia Convention) at physiological pH 7.2. The antimicrobial activity was efficient, showing large reductions in the bacterial population. (See Table 1). The inoculating amount of bacteria was  $\sim 10^7$  organisms and at 4 hours of contact time at 12.5  $\mu\text{g}/\text{ml}$  of V2 dimer, the solution was almost sterilised.

TABLE 1

Antimicrobial activity against <i>P. aeruginosa</i> ATCC 9027 (Log Reduction) in USP Phosphate buffer pH 7.2 at 35° C.			
Test Concentration ug/ml	Diluent for dilution of sample from 100 ug/ml to test concentration		
	USP Phosphate Buffer pH 7.2 4 hrs	USP Phosphate Buffer pH 7.2 4 hrs	USP Phosphate Buffer pH 7.2 4 hrs
25.0	>6.54	>6.38	ND
12.5	>6.54	>6.38	ND
6.25	2.82	1.84	3.04
3.125	0.59	0.22	0.69

The salt solution of the USP buffer used above is dilute compared to physiological conditions, and therefore the antimicrobial activity of the V2 dimer was also tested at physiological salt conditions using 10 mM phosphate buffer pH 7.2. The V2 dimer also maintained high antimicrobial activity against *P. aeruginosa* ATCC 9027 at physiological salt concentration (Table 2).

TABLE 2

Antimicrobial Activity against <i>Pseudomonas aeruginosa</i> ATCC 9027 (Log Reduction) in 10 mM Potassium Phosphate Buffer pH 7.2 at 35° C.	
Test Concentration ug/ml	Diluent for dilution of sample from 100 ug/ml to test concentration 10 mM Potassium Phosphate Buffer
50.0	5.63
25.0	5.63
12.5	2.93
6.25	2.20
3.125	2.00



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In addition, the antimicrobial activity of the V2 dimer was also assayed under high salt concentration of 155.2 mM NaCl. The results indicated that the V2 dimer at concentrations of 12.5 to 50 µg/ml had antimicrobial activity at high salt concentration.

TABLE 3

Antimicrobial Activity against <i>Pseudomonas aeruginosa</i> ATCC 9027 (Log Reduction) at 35° C.			
Test	Diluent for dilution of sample from 100 µg/ml to test concentration		
	155.2 mM NaCl 4 hrs	155.2 mM NaCl 4 hrs	155.2 mM NaCl 4 hrs
Concentration µg/ml			
50.0	ND	5.23	2.82
25.0	2.82	2.78	1.71
12.5	1.71	2.14	0.78
6.25	0.78	ND	ND
3.125	ND	ND	ND

ND—not determined.

## Example 4

## Comparison of the V2 Dimer with hBD3 Derived Peptide Monomers

10 amino acid peptide monomers derived from hBD3 as described previously (WO 2007/126392) were also assayed for their activity against *P. aeruginosa* ATCC 9027.

The antimicrobial properties of the monomers of V2, L2, C2, F2 and H2 in USP phosphate buffer were studied and shown in Table 4.

TABLE 4

Antimicrobial Activity of 10 amino acid peptide monomers against <i>Pseudomonas aeruginosa</i> ATCC 9027 (Log Reduction) at 35° C.						
Concentration ug/ml	V2		L2	C2	F2	H2
	4 hrs	6 hrs	4 hrs	4 hrs	4 hrs	4 hrs
200.00	ND	ND	ND	0.41	0.33	0.29
100.00	ND	ND	0.78	0.57	0.61	0.62
50.00	3.97	5.62	1.15	0.63	0.87	ND
25.00	3.33	ND	1.09	ND	ND	ND
12.5	3.23	ND	0.95	ND	ND	ND
6.25	3.80	ND	1.17	ND	ND	ND
3.125	0.50	ND	1.01	ND	ND	ND
1.558	0.35	ND	0.89	ND	ND	ND

ND—not determined

Note:

0.5 log reduction equals to 68% bacteria being killed; 1 log reduction equals to 90% bacteria being killed; 2 log reduction equals to 99% bacteria being killed; 3 log reduction equals to 99.9% bacteria being killed.

Comparing the results of the V2 dimer in Table 1 to the V2 monomer in Table 4 the V2 dimer shows a much higher efficiency in killing than the V2 monomer. The V2 dimer showed a >6 log reduction (Table 1) at a concentration of 12.5 µg/ml at 4 hours compared to the V2 monomer which shows a 3.23 log reduction at 12.5 µg/ml and only a 3.97 log reduction at 50 µg/ml (Table 4).

## Example 5

Antimicrobial Activity of V2 Dimer Against Clinical Isolates of *P. aeruginosa*

The antimicrobial activity of the V2 dimer was also tested against several clinical isolates of *P. aeruginosa* from the

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sputum, wound, urine and the eye. The V2 dimer also showed antimicrobial activity against these clinical isolates (See Tables 5-8), suggesting that the V2 primer may be effectively used against actual clinical specimens.

TABLE 5

Antimicrobial activity of V2 dimer against clinical strains of <i>P. aeruginosa</i> (Log Reduction) isolated from sputum at 35° C.			
Test	Diluent for dilution of sample from 100 µg/ml to test concentration		
	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2
Type of Clinical <i>Pseudomonas aeruginosa</i>			
Test	PA1-PAE 230 DR4877/07	PA1-PAE 230 DR4877/07	PA1-PAE 230 DR4877/07
Concentration ug/ml	Source: Sputum 4 hrs	Source: Sputum 4 hrs	Source: Sputum 4 hrs
50.0	4.53	>5.52	>5.49
25.0	4.44	5.22	>5.49
12.5	3.02	3.65	5.07
6.25	2.21	2.41	3.12
3.125	1.49	1.99	2.41

TABLE 6

Antimicrobial activity of V2 dimer against clinical strains of <i>P. aeruginosa</i> (Log Reduction) isolated from wound at 35° C.			
(A) Data for <i>P. aeruginosa</i> PAE239 DM5790/07			
Test	Diluent for dilution of sample from 100 µg/ml to test concentration		
	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2
Type of Clinical <i>Pseudomonas aeruginosa</i>			
Test Concentration ug/ml	PA2-PAE 239 DM5790/07 Source: Wound 4 hrs	PA2-PAE 239 DM5790/07 Source: Wound 4 hrs	PA2-PAE 239 DM5790/07 Source: Wound 4 hrs
50.0	1.35	1.58	1.97
25.0	1.21	1.56	1.40
12.5	1.18	1.52	1.26
6.25	1.12	1.29	1.22
3.125	1.16	1.27	1.22
(B) Data for <i>P. aeruginosa</i> PAE249 DM15013			
Test	Diluent for dilution of sample from 100 µg/ml to test concentration		
	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2
Type of Clinical <i>Pseudomonas aeruginosa</i>			
Test Concentration ug/ml	PA4-PAE 249 DM15013 Source: Wound 4 hrs	PA4-PAE 249 DM15013 Source: Wound 4 hrs	PA4-PAE 249 DM15013 Source: Wound 4 hrs
50.0	5.08	4.57	>4.99
25.0	4.69	4.34	>4.99

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TABLE 6-continued

Antimicrobial activity of V2 dimer against clinical strains of <i>P. aeruginosa</i> (Log Reduction) isolated from wound at 35° C.			
12.5	3.96	4.19	4.60
6.25	3.13	2.41	3.18
3.125	2.12	2.00	1.71

TABLE 7

Antimicrobial activity of V2 dimer against clinical strains of <i>P. aeruginosa</i> (Log Reduction) isolated from urine at 35° C.			
Test Concentration ug/ml	Diluent for dilution of sample from 100 ug/ml to test concentration		
	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2
	Type of Clinical <i>Pseudomonas aeruginosa</i>		
	PA3-PAE 240 DU14476/07 Source: Urine 4 hrs	PA3-PAE 240 DU14476/07 Source: Urine 4 hrs	PA3-PAE 240 DU14476/07 Source: Urine 4 hrs
50.0	2.08	1.88	1.72
25.0	1.58	1.52	1.56
12.5	1.21	1.52	1.42
6.25	1.25	1.52	1.28
3.125	1.15	1.52	1.13

TABLE 8

Antimicrobial activity of V2 dimer against <i>P. aeruginosa</i> (Log Reduction) isolated from the eye at 35° C. IN USP phosphate buffer		
(A) Data for <i>P. aeruginosa</i> 07DM02357		
Test Concentration ug/ml	Diluent for dilution of sample from 100 ug/ml to test concentration	
	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2
	Type of Clinical <i>Pseudomonas aeruginosa</i>	
	Ps-A 07DM02357 Source: Eye 4 hrs	Ps-A 07DM02357 Source: Eye 4 hrs
50.0	2.41	5.53
25.0	1.76	3.66
12.5	1.75	3.17
6.25	1.60	2.35
3.125	1.37	1.94
(B) Data for <i>P. aeruginosa</i> 07DM023376		
Test Concentration ug/ml	Diluent for dilution of sample from 100 ug/ml to test concentration	
	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2
	Type of Clinical <i>Pseudomonas aeruginosa</i>	
	Ps-B 07DM023376 Source: Eye 4 hrs	Ps-B 07DM023376 Source: Eye 4 hrs
50.0	2.39	2.25
25.0	1.81	2.18
12.5	1.76	2.15

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TABLE 8-continued

Antimicrobial activity of V2 dimer against <i>P. aeruginosa</i> (Log Reduction) isolated from the eye at 35° C. IN USP phosphate buffer			
6.25	1.38	1.88	
3.125	1.07	1.16	
(C) Data for <i>P. aeruginosa</i> 07DM023155			
Test Concentration ug/ml	Diluent for dilution of sample from 100 ug/ml to test concentration		
	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2
	Type of Clinical <i>Pseudomonas aeruginosa</i>		
	Ps-C 07DM023155	Ps-C 07DM023155	Ps-C 07DM023155
	Source: Eye	Source: Eye	Source: Eye
	4 hrs	4 hrs	4 hrs
50.0	4.97	3.58	4.30
25.0	3.14	3.23	3.52
12.5	2.77	3.21	2.89
6.25	2.71	2.23	2.77
3.125	1.91	2.00	2.33
(D) Data for <i>P. aeruginosa</i> 07DM023104			
Test Concentration ug/ml	Diluent for dilution of sample from 100 ug/ml to test concentration		
	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2	
	Type of Clinical <i>Pseudomonas aeruginosa</i>		
	Ps-D 07DM023104	Ps-D 07DM023104	
	Source: Eye	Source: Eye	
	4 hrs	4 hrs	
50.0	3.45	4.53	
25.0	3.42	3.92	
12.5	2.82	3.48	
6.25	2.68	2.89	
3.125	1.71	2.27	

The antimicrobial activity of V2 dimer was also tested for clinical isolates from at physiological salt conditions using 10 mM phosphate buffer. V2 dimer also showed antimicrobial activity against clinical isolates from the eye in repeated experiments (Table 9A, 9B and 9C), the wound, sputum and urine in repeated experiments (Table 10A and B), suggesting that the V2 dimer can also be used against clinical isolates under physiological conditions.

TABLE 9

Antimicrobial activity of V2 dimer against <i>P. aeruginosa</i> (Log Reduction) isolated from the eye at 35° C. IN 10 mM potassium phosphate buffer at 35° C.				
(A)				
Diluent for dilution of sample from 100 ug/ml to test concentration				
Type of Clinical <i>Pseudomonas aeruginosa</i>				
Test Concentration ug/ml	10 mM Sodium Phosphate Buffer	10 mM Phosphate Buffer	10 mM Sodium Phosphate Buffer	10 mM Sodium Phosphate Buffer
	Ps-A 07DM02357 Source: Eye 4 hrs	Ps-B 07DM023376 Source: Eye 4 hrs	Ps-C 07DM023155 Source: Eye 4 hrs	Ps-D 07DM023104 Source: Eye 4 hrs
50.0	2.92	2.23	3.47	3.77
25.0	2.05	1.87	3.14	2.09
12.5	1.04	1.29	1.60	1.47
6.25	0.97	1.18	1.62	1.04
3.125	0.56	0.45	0.94	0.61
(B)				
Diluent for dilution of sample from 100 ug/ml to test concentration				
Type of Clinical <i>Pseudomonas aeruginosa</i>				
Test Concentration ug/ml	10 mM Potassium Phosphate Buffer	10 mM Potassium Phosphate Buffer	10 mM Potassium Phosphate Buffer	10 mM Potassium Phosphate Buffer
	Ps-A 07DM02357 Source: Eye 4 hrs	Ps-B 07DM023376 Source: Eye 4 hrs	Ps-C 07DM023155 Source: Eye 4 hrs	Ps-D 07DM023104 Source: Eye 4 hrs
50.0	1.77	1.51	3.06	1.54
25.0	1.36	1.45	2.99	1.49
12.5	0.78	1.35	1.68	1.19
6.25	0.69	1.12	1.41	1.09
3.125	0.62	0.76	0.79	0.73
(C)				
Diluent for dilution of sample from 100 ug/ml to test concentration				
Type of Clinical <i>Pseudomonas aeruginosa</i>				
Test Concentration ug/ml	10 mM Potassium Phosphate Buffer	10 mM Potassium Phosphate Buffer	10 mM Potassium Phosphate Buffer	10 mM Potassium Phosphate Buffer
	Ps-B 07DM023376 Source: Eye 4 hrs	Ps-C 07DM023155 Source: Eye 4 hrs	Ps-D 07DM023104 Source: Eye 4 hrs	
50.0	2.28	2.62	1.39	
25.0	1.90	2.33	0.97	
12.5	0.95	1.70	0.46	
6.25	1.01	1.14	0.13	
3.125	0.64	0.09	-0.54	

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TABLE 10

Antimicrobial activity of V2 dimer against clinical isolated <i>P. aeruginosa</i> (Log Reduction) isolated from wound, sputum and urine at 35° C. IN 10 mM potassium phosphate buffer				
(A)				
Diluent for dilution of sample from 100 ug/ml to test concentration				
	10 mM Potassium Phosphate Buffer	10 mM Potassium Phosphate Buffer	10 mM Potassium Phosphate Buffer	10 mM Potassium Phosphate Buffer
Type of Clinical <i>Pseudomonas aeruginosa</i>				
	PA1-PAE 230	PA2-PAE 239	PA3-PAE 240	PA4-PAE 249
Test Concentration ug/ml	DR4877/07 Source: Sputum 4 hrs	DM5790/07 Source: Wound 4 hrs	DU14476/07 Source: Urine 4 hrs	DM15013 Source: Wound 4 hrs
50.0	2.54	1.21	1.28	3.01
25.0	2.03	0.78	1.11	2.02
12.5	1.14	0.58	0.72	1.30
6.25	0.66	0.40	0.61	0.97
3.125	0.21	0.06	0.29	0.26
(B)				
Diluent for dilution of sample from 100 ug/ml to test concentration				
	10 mM Potassium Phosphate Buffer	10 mM Potassium Phosphate Buffer	10 mM Potassium Phosphate Buffer	10 mM Potassium Phosphate Buffer
Type of Clinical <i>Pseudomonas aeruginosa</i>				
	PA1-PAE 230	PA1-PAE 230	PA2-PAE 239	PA2-PAE 239
Test Concentration ug/ml	DR4877/07 Source: Sputum 4 hrs	DR4877/07 Source: Sputum 4 hrs	DM5790/07 Source: Wound 4 hrs	DM5790/07 Source: Wound 4 hrs
50.0	2.54	2.36	1.21	1.59
25.0	2.03	1.97	0.78	1.27
12.5	1.14	1.26	0.58	0.77
6.25	0.66	0.97	0.40	0.62
3.125	0.21	0.64	0.06	0.49

## Example 6

## Comparison of Antimicrobial Activity of V2 Dimer and Gentamicin

A comparison of the antimicrobial activity of V2 dimer and gentamicin was made against clinical isolates of *Pseudomonas aeruginosa*.

In the first study, both V2 dimer and gentamicin were tested against clinical isolates from wound and sputum and compared (See Table 5). The study showed that for the wound isolate, V2 dimer has comparable activity to gentamicin. For the sputum isolate, V2 dimer showed higher antimicrobial activity.

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TABLE 11

Comparison of the antimicrobial activity (Log Reduction) V2 dimer with gentamicin against clinical <i>P. aeruginosa</i> isolates from sputum and wound at 35° C.:				
V2-dimer V2-dimer Gentamicin Gentamicin				
Diluent for dilution of sample from 100 ug/ml to test concentration				
	USP Phosphate Buffer	USP Phosphate Buffer	USP Phosphate Buffer	USP Phosphate Buffer
Type of Clinical <i>Pseudomonas aeruginosa</i>				
	PA1-PAE 230	PA2-PAE 239	PA1-PAE 230	PA2-PAE 239
Test Concentration ug/ml	DR4877/07 Source: Sputum 4 hrs	DM5790/07 Source: Wound 4 hrs	DR4877/07 Source: Sputum 4 hrs	DM5790/07 Source: Wound 4 hrs
50.0	>4.79	2.52	0.39	3.31
25.0	>4.79	2.69	0.54	3.04
12.5	>4.79	2.36	0.65	3.03
6.25	>4.79	2.28	0.35	3.07
3.125	>4.79	2.04	0.22	3.02

In a second study, the antimicrobial activity of both V2 dimer and gentamicin were tested against clinical isolates from urine and sputum and compared (See Table 6). The V2 dimer also showed comparable activity to gentamicin against these two isolates.

TABLE 12

Comparison of the antimicrobial activity (Log Reduction) V2 dimer with gentamicin against clinical <i>P. aeruginosa</i> isolates from urine and wound at 35° C.:				
V2-dimer V2-dimer Gentamicin Gentamicin				
Diluent for dilution of sample from 100 ug/ml to test concentration				
	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2
Type of Clinical <i>Pseudomonas aeruginosa</i>				
	PA3-PAE 240	PA4-PAE 249	PA3-PAE 240	PA4-PAE 249
Test Concentration ug/ml	DU14476/07 Source: Urine 4 hrs	DM15013 Source: Wound 4 hrs	DU14476/07 Source: Urine 4 hrs	DM15013 Source: Wound 4 hrs
50.0	1.81	5.19	4.57	2.63
25.0	1.51	3.86	4.18	2.60
12.5	1.40	3.75	3.88	2.50
6.25	1.22	3.73	3.76	2.41
3.125	1.11	3.77	3.66	2.28

In a third study, the antimicrobial activity of both V2 dimer and gentamicin were tested against clinical isolates from the eye (See Table 13). This study shows that V2 dimer and gentamicin have comparable activity against *P. aeruginosa* isolates from the eye.

TABLE 13

Comparison of the antimicrobial activity (Log Reduction) V2 dimer with gentamicin against clinical <i>P. aeruginosa</i> isolates from urine and wound at 35° C.:				
	V2-dimer	V2-dimer Diluent for dilution of sample from 100 ug/ml to test concentration	Gentamicin	Gentamicin
	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2	USP Phosphate Buffer pH 7.2
	Type of Clinical <i>Pseudomonas aeruginosa</i>			
Test Concentration ug/ml	Ps-C 07DM023155 Source: Eye 4 hrs	Ps-D 07DM023104 Source: Eye 4 hrs	Ps-C 07DM023155 Source: Eye 4 hrs	Ps-D 07DM023104 Source: Eye 4 hrs
50.0	4.31	>4.33	>4.61	>4.33
25.0	3.80	>4.33	>4.61	>4.33
12.5	3.17	4.02	>4.61	>4.33
6.25	3.11	3.75	>4.61	>4.33
3.125	2.74	3.35	>4.61	>4.33

Taken together, the above studies suggest that V2 dimer and gentamicin have comparable antimicrobial activity and the V2 dimer may be used with an efficacy equivalent to gentamicin against microorganisms.

#### Example 7

#### Antimicrobial Activity of V2 Dimer Against Other Organisms

##### (A) *Candida albicans* ATCC 10231

The antimicrobial activity of V2 dimer against *Candida albicans* ATCC 10231 was tested. *C. albicans* at  $7.2 \times 10^5$  CFU in USP phosphate buffer were mixed with different concentrations of V2 dimer and incubated for 4 hours at 35° C. The results show that V2 dimer at 50 ug, 25 ug and 12.5 ug/ml achieved 3.6 to 4 log reductions against *C. albicans* ATCC 10231. (Table 14)

TABLE 14

Antimicrobial Activity against <i>Candida albicans</i> ATCC 10231 (Log Reduction) at 35° C.		
Test Concentration of V2 dimer (ug/ml)	Time (hours)	Mean Log reduction
50	4	3.94
25	4	4.01
12.5	4	3.62
6.25	4	2.30
3.125	4	0.71

##### (B) *Bacillus cereus* ATCC 11778

The antimicrobial activity of V2 dimer against *Bacillus cereus* ATCC 11778 was tested. V2 dimer showed antimicrobial activity against *B. cereus* (Table 15)

TABLE 15

Antimicrobial Activity against <i>Bacillus cereus</i> ATCC 11778 (Log Reduction) at 35° C.		
	Diluent for dilution of sample from 100 ug/ml to test concentration	
Test Concentration ug/ml	USP Phosphate Buffer pH 7.2 4 hours	USP Phosphate Buffer pH 7.2 4 hours
50.0	2.29	1.70
25.0	2.76	2.04
12.5	2.75	1.32
6.25	0.86	1.26

##### (C) Clinical *Escherichia coli* DB0016027R

In addition, the V2 dimer was able to produce reductions against the multiple antibiotic resistant strain of *E. coli* DB0016027R (clinically isolated from blood) which has an antibiogram profile of resistance to gentamicin, ampicillin and other antibiotics.

TABLE 16

Antimicrobial Activity against <i>E. coli</i> DB0016027R (Log Reduction) at 35° C.	
Test Concentration ug/ml	Diluent for dilution of sample from 100 ug/ml to test concentration USP Phosphate Buffer pH 7.2 4 hours
50.0	2.83
25.0	2.52
12.5	2.68
6.25	2.45
3.125	2.60

V2 dimer's antimicrobial property against *C. albicans* and other bacteria suggests that it may be an effective broad spectrum antimicrobial. Further, V2 dimer's efficacy against a multiple antibiotic resistant strain of *E. coli* suggests that it may be an effective therapeutic agent against where other antibiotics cannot be used.

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## Example 8

## Cytotoxicity of V2 Dimer

The cytotoxicity of V2 dimer were tested against human conjunctival cells according to the method described in WO 2007/126392 and compared with native hBD3. FIG. 4 shows that V2 dimer has reduced cytotoxicity to human conjunctival cells in comparison with wildtype hBD3. Wildtype hBD3 was cytotoxic at concentrations of about 15 µg/ml, however, the V2 dimer was not cytotoxic at concentrations of 100 µg/ml. The cytotoxicity profile of the V2 dimer was comparable to that of the monomer peptides from WO 2007/126392

The reduced cytotoxicity of V2 dimer to human conjunctival cells and the high antimicrobial activity of the V2 dimer suggest that this dimer could be used in a host for treating microbial infections and/or reducing the growth of microorganism in the host. Other dimers of the peptides from WO 2007/126392 are similarly expected to also show high antimicrobial activity and low cytotoxicity.

## Example 9

Minimum Inhibitory Concentration (MIC)  
Determination by Broth Macrodilution Technique

MICs was determined by broth macrodilution method modified from that described by the National Committee for Clinical Laboratory Standards (NCCL). The Mueller Hinton Broth (MHB) at 1/2 strength and without addition of Ca<sup>2+</sup> and Mg<sup>2+</sup> was used for dilution. Serial twofold dilutions of V2 dimer solution was prepared in MHB (1/2 strength) in test tubes. 1 ml of inoculum of test organisms in MHB (1/2 strength) was added to 1 ml of each dilution of V2 dimer to yield as final concentration of 10<sup>4</sup> to 10<sup>5</sup> colony forming units/ml in each test tube. The tubes were incubated at 35° C. for 16 to 20 hours. A positive control containing only the broth and organism, and a negative control tube containing only the broth were also incubated in parallel to the test samples. The above was repeated for each different test organism in separate experiments. The MIC of V2 dimer peptide for each clinical isolate or reference organism was read as the lowest concentration of peptide that inhibited visible growth of the test organism.

The MIC was also determined for other peptides and compared to gentamicin. The MIC results are shown in Tables 17-20.

TABLE 17

MIC (ug/ml) results of V2-dimer, Y2-dimer, W2-dimer and gentamicin against different strains of Gram-negative bacteria <i>Pseudomonas aeruginosa</i> and <i>Escherichia coli</i>					
Test Organism	V2-dimer	Y2-dimer	W2-dimer	V2-tetramer	Gentamicin
<i>Pseudomonas aeruginosa</i> ATCC 27853	12.5				
Clinical <i>Pseudomonas aeruginosa</i> DM 023104 Source: Eye	12.5	12.5	12.5	6.25	
Clinical <i>Pseudomonas aeruginosa</i> DM 023155 Source: Eye	12.5	25	12.5	6.25	
Clinical <i>Pseudomonas aeruginosa</i> 07DM 023257 Source: Eye	12.5	12.5	12.5	12.5	

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TABLE 17-continued

MIC (ug/ml) results of V2-dimer, Y2-dimer, W2-dimer and gentamicin against different strains of Gram-negative bacteria <i>Pseudomonas aeruginosa</i> and <i>Escherichia coli</i>					
Test Organism	V2-dimer	Y2-dimer	W2-dimer	V2-tetramer	Gentamicin
Clinical <i>Pseudomonas aeruginosa</i> 07DM023376 Source: Eye	12.5	25	25	6.25	
Clinical <i>Pseudomonas aeruginosa</i> PAE 230 DR4877/07 Source: Sputum	12.5	12.5	12.5	12.5	400
Clinical <i>Pseudomonas aeruginosa</i> PAE 239 DM5790/07 Source: Wound	12.5	12.5	12.5	12.5	25
Clinical <i>Pseudomonas aeruginosa</i> PAE 240 DU14476/07 Source: Urine	6.25	6.25	12.5	6.25	
Clinical <i>Pseudomonas aeruginosa</i> PAE 249 DM15013 Source: Wound	6.25	12.5	6.25	6.25	
<i>Escherichia coli</i> ATCC25922	12.5	12.5	25	12.5	
Clinical <i>Escherichia coli</i> DB16027 Source: Blood	6.25	6.25	12.5	6.25	0.78
Clinical <i>Escherichia coli</i> DU46381R Source: Urine	6.25	12.5	6.25	6.25	

TABLE 18

MIC (ug/ml) results of V2-dimer, Y2-dimer, W2-dimer and Gentamicin against different strains of Gram-positive bacteria <i>Staphylococcus aureus</i> , <i>Bacillus cereus</i> and fungi <i>Candida albicans</i> , <i>Fusarium solani</i>					
Test Organism	V2-dimer	Y2-dimer	W2-dimer	V2-tetramer	Gentamicin
Methicillin-resistant <i>staphylococcus aureus</i> (MRSA) DM09808R Source: Eye	12.5				12.5
Clinical <i>Staphylococcus aureus</i> DM4001 Source: Eye	12.5	12.5	12.5	12.5	
<i>Bacillus cereus</i> ATCC 11778	12.5				
<i>Candida albicans</i> ATCC10231	12.5	25	12.5	12.5	
Clinical <i>Candida albicans</i> DF2672R Source: Urine	12.5	12.5	12.5	12.5	
<i>Fusarium solani</i> ATCC 36031	25	12.5	12.5	3.125	

TABLE 19

MIC (ug/ml) results of several C-terminus monomers against different strains of Gram-negative bacteria <i>Pseudomonas aeruginosa</i> and <i>Escherichia coli</i>					
Test Organism	Y2-8AA	V2-8AA	W2-8AA	V4 monomer	Y4 monomer
<i>Pseudomonas aeruginosa</i> ATCC 27853					
Clinical <i>Pseudomonas aeruginosa</i> DM 023104 Source: Eye	>50	>50	25	>50	50

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TABLE 19-continued

MIC (ug/ml) results of several C-terminus monomers against different strains of Gram-negative bacteria <i>Pseudomonas aeruginosa</i> and <i>Escherichia coli</i>					
Test Organism	Y2- 8AA	V2- 8AA	W2- 8AA	V4 monomer	Y4 monomer
Clinical <i>Pseudomonas aeruginosa</i> DM 023155 Source: Eye	>50	>50	50	>50	25
Clinical <i>Pseudomonas aeruginosa</i> 07DM 023257 Source: Eye	>50	>50	>50	>50	>50
Clinical <i>Pseudomonas aeruginosa</i> 07DM023376 Source: Eye	50	>50	25	>50	>50
Clinical <i>Pseudomonas aeruginosa</i> PAE 230 DR4877/07 Source: Sputum	>50	>50	>50	>50	>50
Clinical <i>Pseudomonas aeruginosa</i> PAE 239 DM5790/07 Source: Wound	>50	>50	>50	>50	50
Clinical <i>Pseudomonas aeruginosa</i> PAE 240 DU14476/07 Source: Urine	6.25	25	12.5	>50	25
Clinical <i>Pseudomonas aeruginosa</i> PAE 249 DM15013 Source: Wound	>50	>50	50	>50	50
<i>Escherichia coli</i> ATCC25922	25	>50	12.5	>50	12.5
Clinical <i>Escherichia coli</i> DB16027 Source: Blood	25	>50	6.25	>50	6.25
Clinical <i>Escherichia coli</i> DU46381R Source: Urine	25	50	6.25	>50	12.5

TABLE 20

MIC (ug/ml) results of several C-terminus monomers against different strains of Gram-positive bacteria <i>Staphylococcus aureus</i> , <i>Bacillus cereus</i> and fungi <i>Candida albicans</i> , <i>Fusarium solani</i>					
Test Organism	Y2- 8AA	V2- 8AA	W2- 8AA	V4 monomer	Y4 monomer
Methicillin-resistant <i>staphylococcus aureus</i> (MRSA) DM09808R Source: Eye	>50	>50	12.5	>50	12.5
Clinical <i>Staphylococcus aureus</i> DM4001 Source: Eye					
<i>Bacillus cereus</i> ATCC 11778					
<i>Candida albicans</i> ATCC10231	25	>50	12.5	25	12.5
Clinical <i>Candida albicans</i> DF2672R Source: Urine	50	>50	12.5	>50	25
<i>Fusarium solani</i> ATCC 36031	50	>100	25	>100	12.5

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Example 10

### Determination of Minimum Inhibitory Concentration of B2088 and B2088\_99

The minimum inhibitory concentration (MIC) was determined using a broth microdilution method. Different strains of bacterial cells were grown in Mueller Hinton Broth (MHB) overnight. 100 µl of adjusted inoculum in MHB was added to 100 µl of each dilution of peptide or antibiotics dissolved in the broth, so as to yield a final cell density of 10<sup>5</sup> to 10<sup>6</sup> cfu/ml in each well of a microtitre plate. Additional microtitre plates may be used as necessary, if more wells are required for the assay or duplicate plates are required. The microtitre plates were incubated at 35° C. for 24 h and the absorbance at 600 nm (OD<sub>600</sub>) was monitored every 30 minutes. The plates were incubated at 35° C. for 24 h and the absorbance at 600 nm (OD<sub>600</sub>) was monitored every 30 minutes. A positive control well contained the broth and organisms (no peptides/antibiotics), and a negative control well contains only the broth. The MIC of peptides for each clinical isolate or reference organism was recorded as the lowest concentration of peptide/antibiotic that inhibited visible growth of the test organism. contains only the broth. The MIC of peptides for each clinical isolate or reference organism was recorded as the lowest concentration of peptide/antibiotic that inhibited visible growth of the test organism.

Two dimers B2088 (V2 dimer) and B2088-99 were tested and the results are shown in Table 21.

B2088\_99 corresponds to the dimer (RGRKGGRR)<sub>2</sub>KK or (SEQ ID NO: 59)<sub>2</sub>KK.

The results are shown in Table 21.

TABLE 21

Minimum inhibitory concentration (MIC) of B2088 and B2088_99		
Strains <sup>a</sup>	MIC in µM of	
	B2088	B2088_99
Pa DR 18531	2.73	2.9
Pa DM 4150R	2.73	5.8
Pa DR 23257	2.73	5.8
Pa DR 23376	5.46	5.8
Pa DR 14476	5.46	5.8
Pa DR 23155	2.7	2.9
Pa DR 5790	10.91	5.8
Pa DR 4877	2.7	2.9
Pa ATCC 9027	2.7	2.9
Pa 23155	2.7	2.9
Pa ATCC 27853	2.7	2.9
Kp ATCC 10031	1.4	1.5
Kp 4299	2.7	2.9

<sup>a</sup>Pa is *Pseudomonas aeruginosa* and Kp is *Klebsiella pneumoniae*

### Example 11

#### LPS, Lipid A and Mg<sup>2+</sup> Studies

Bodipy TR Cadavarine (BC) displacement assay for LPS and lipid a binding to branched peptides: BC forms tight complexes with LPS/lipid which results in quenching of its fluorescence intensity. When peptides/molecules that can

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interact with LPS/lipid are added, BC will be displaced from the complex with concomitant dequenching of its fluorescence. The BC displacement assay was carried out in 5 mM HEPES buffer (pH 7.0). 10  $\mu$ M of the dye was added to LPS or lipid A in a stirred quartz cuvette. Fluorescence measurements were performed using an excitation wavelength of 580 nm and the emission intensity at 620 nm was monitored. The displacement assay was performed by the addition of various concentrations of peptides. Polymyxin B was used as a positive control. BC occupancy was calculated using the equation:

$$OF = F_0 - F / F_0 - F_{max}$$

Where  $F_0$  is the fluorescence intensity of free BC,  $F_{max}$  is fluorescence intensity of LPS-BC complex and  $F$  is fluorescence intensity after the addition of peptides or polymyxin B.

The results are shown in FIGS. 10(A) and (B). The BC displacement assay suggested that B2088 binds 2 times more strongly to LPS and >10 times more strongly to Lipid A than B2088\_99.

Effect of LPS and  $Mg^{2+}$  on MIC: To examine the interaction of peptide and LPS, the concentration of the latter was added exogenously from (0.001-100 mg/ml) in MHB at a peptide concentration of 1xMIC. The % inhibition was estimated and the amount of LPS required for 50% of antibacterial activity ( $IC_{50}$ ) was determined.

The results are shown in FIG. 10(C) and suggest that B2088\_99 requires a higher concentration of LPS to suppress the inhibitory activity compared to B2088. These results further confirm weaker binding of B2088\_99 to B2088.

To study the effect of  $Mg^{2+}$  on the MIC of branched peptides, the concentration of  $MgCl_2$  was varied in MHB and the MIC was determined as before. The effect of  $Mg^{2+}$  on the MIC of B2088 and B2088\_99 are shown in FIG. 10(D).  $Mg^{2+}$  stabilises outer membrane of Gram-negative bacteria and antagonizes the permeability of cationic agents. The strong dependency of MIC on  $Mg^{2+}$  concentration for B2088 suggests that the latter binds to the lipid A portion of OM more strongly than B2088\_99.

### Example 12

#### Bacterial Viability Assay

Cell viability was conducted for two Gram-negative bacteria (Pa 9027 and Pa 27853). The cultures were grown overnight on trypticase soy (TS) agar and a few isolated colonies were inoculated to achieve a turbidity equivalent to 0.5 McFarland standard. The cell concentration was adjusted to 10<sup>6</sup> CFU/ml with 10 mM phosphate buffer and separated into different tubes to get final concentration of 10<sup>5</sup> CFU/ml. The peptides were added to the individual tubes to obtain concentrations of 1/8 MIC, 1/4 MIC, 1/2 MIC, 1 MIC, 2 MIC and 4 MIC. The tubes were incubated at 37° C. for 22 h. Serial dilution was performed and 100  $\mu$ L of the suspension was aliquot onto Mueller Hinton Agar (MHA) plate. The plates were incubated for 24 h at 37° C. for colony counting. Positive control was performed at 0h and 22h.

The results are shown in Table 22 and FIG. 11. The effective dose of peptide that reduces the viability of bacteria cells by 50% ( $ED_{50}$ ) is two times lower for B2088\_99 than B2088.

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TABLE 22

Bactericidal properties of B2088 and B2088_99 as measured by $ED_{50}$ (Effective dose to kill 50% of bacterial cells)		
Strains	$ED_{50}$ in $\mu$ M of	
	B2088	B2088_99
PA 9027	0.7 $\pm$ 0.02	0.33 $\pm$ 0.05
PA 28753	1.1 $\pm$ 0.04	0.48 $\pm$ 0.03

### Example 13

#### Time-Kill Kinetics Assay

The kinetics of bactericidal action was performed by the assay reported before. Briefly, few colonies of overnight grown *P. aeruginosa* strains were collected from tryptic soy agar plate and suspended in US pharmacopeia phosphate buffer (pH 7.2). The suspension was adjusted to an initial inoculum of 10<sup>6</sup> CFU/mL and incubated with various concentrations of B2088 and B2088\_99 at 35° C. 0.1 ml aliquots were withdrawn at various time intervals, diluted 10<sup>2</sup>-10<sup>4</sup> fold using the same buffer, plated on tryptic soy agar plates and incubated at 35° C. The colonies were counted after 24 h incubation and expressed as CFU/ml. Buffer without peptides served as a positive control and % bacterial viability is estimated using the following equation:

$$\text{Bacterial viability} = 1 - (\text{CFU/mL})_{\text{peptide}} / (\text{CFU/mL})_{\text{control}} \times 100$$

The results are shown in FIG. 12. B2088\_99 displayed faster kill kinetics against both the *Pseudomonas* strains at 1x and 2xMIC.

### Example 13

#### Outer Membrane (OM) Permeability Assay

The membrane impermeable probe N-phenyl-1-naphthyl amine (NPN) was used to probe the OM permeability of peptides. An overnight culture of *P. aeruginosa* ATCC 9027 was harvested by centrifugation (3000 rpm, 4° C.). The pellet was washed 2 times and resuspended in 5 mM HEPES buffer (pH 7.2) to OD<sub>600</sub> of 0.4. The cells were placed in a 10 mm stirred cuvette and NPN was added to a final concentration of 10  $\mu$ M. Appropriate concentrations of B2088 was added and the increase in fluorescence intensity was monitored on a Quanta Master fluorescence spectrophotometer (Photon Technology International, New Jersey, USA). The excitation and emission wavelengths were set at 350 and 410 nm with slit widths at 2 and 5 nm, respectively. The % NPN uptake was calculated relative to the increase in NPN fluorescence intensity after the addition of 50  $\mu$ M polymyxin B.

The results are shown in FIG. 13. The peptide concentration required to cause 50% increase in the NPN fluorescence intensity ( $PC_{50}$ ) was measured.  $PC_{50}$  for B2088\_99 was higher than B2088, indicating that the latter had a better OM permeability.

Based on the above results of Examples 10-13, replacement of hydrophobic valine residues in B2088 lead to better bactericidal and kill-kinetics properties but compromised OM permeability and strong lipid A binding. To confirm these results, the FICI for B2088 and B2088\_99 with various classes of antibiotics was determined.



## Determination of Fractional Inhibitory Concentration Index (FICI)

For the determination of FICI of peptides in combination with other antibiotics, the multidrug resistant strain, *P. aeruginosa* DR4877 was used. Prior to the testing, stock solutions of each drug (multivalent peptides and antibiotics) to at least 2×MIC are prepared in Mueller-Hinton broth (MHB). The checkerboard was assembled on a 96-well microtitre plate by overlaying serial two-fold dilutions of multivalent peptides perpendicularly (i.e. along the ordinate) to serial dilutions of antibiotics (i.e., along the abscissa). Each well consisted of 100 µL serially diluted peptide or antibiotics alone and in combinations in MHB and 100 µL inoculum (OD<sub>600</sub>=0.08). The microtitre plate was incubated at 35° C. for 24 h. Inhibition was determined both by visual examination and by OD<sub>600</sub> measurements. The fractional inhibitory concentration indices will be calculated using the equation,

$$FIC_{index} = \frac{MIC_{peptide}^{comb}}{MIC_{peptide}} + \frac{MIC_{antibiotics}^{comb}}{MIC_{antibiotics}}$$

The FIC indices used to characterize antibiotic combinations as follows: FIC index<0.5 synergistic; additivity, 0.5<FIC index>1.0; indifference, 1<FIC index<4; FIC index>4, antagonism. We will compare the synergistic action of the peptides with polymyxin B as a standard.

The results are shown in Table 23. B2088 displayed better synergism than B2088\_99 or B2088 has better sensitising ability for various antibiotics against multidrug resistant *P. aeruginosa* compared to B2088\_99.

TABLE 23

Synergies between B2088 and B2088_99 with various classes of antibiotics.			
Antibiotics	Class	FIC Index	
		B2088	B2088_99
Carbenicillin	Penicillin	0.5	0.63
Chlormphenicol		0.38	0.38
Erythromycin	Macrolides	0.5	0.63
Nalidixic acid	Quinolones	0.56	0.63
Gatifloxacin	Fluoroquinolones	0.56	0.75
Imipenem	Carbapenems	0.5	0.63
Kanamycin	Aminoglycosides	0.5	0.5
Streptomycin	Aminoglycosides	0.63	0.75

## Animal Model of Infection

To confirm the in vitro results that B2088 has a better synergistic antimicrobial activity compared to B2088\_99, the effect of these two peptides in a mouse model of corneal infection was compared. For the animal model of infection, *P. aeruginosa* ATCC 9027 was used. The results are shown in FIG. 14. At 0.5 mg/ml of B2088 in combination with ½ the dose of gatifloxacin, a complete sterilisation of the infection was observed and the activity was superior to B2088\_99.

In summary, it was found that B2099 acts at submicrogram, sub MIC values to increase the activity of existing antibiotics even on resistant forms of *Pseudomonas*.

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## SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 59

<210> SEQ ID NO 1

<211> LENGTH: 45

<212> TYPE: PRT

<213> ORGANISM: Homo sapien

<400> SEQUENCE: 1

Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Cys Arg Val Arg Gly Gly  
1 5 10 15

Arg Cys Ala Val Leu Ser Cys Leu Pro Lys Glu Glu Gln Ile Gly Lys

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20                      25                      30  
 Cys Ser Thr Arg Gly Arg Lys Cys Cys Arg Arg Lys Lys  
          35                      40                      45  
  
 <210> SEQ ID NO 2  
 <211> LENGTH: 45  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived peptide, C replaced with X  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (11)..(41)  
 <223> OTHER INFORMATION: X comprises any amino acid  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (44)..(45)  
 <223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 2  
  
 Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Xaa Arg Val Arg Gly Gly  
 1                      5                      10                      15  
  
 Arg Xaa Xaa Val Leu Ser Ala Leu Pro Lys Glu Glu Gln Ile Gly Lys  
          20                      25                      30  
  
 Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa  
          35                      40                      45

<210> SEQ ID NO 3  
 <211> LENGTH: 43  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 fragment excluding terminal KK, C residues  
                                  replaced with X  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (11)..(41)  
 <223> OTHER INFORMATION: X comprises any amino acid

<400> SEQUENCE: 3  
  
 Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Xaa Arg Val Arg Gly Gly  
 1                      5                      10                      15  
  
 Arg Xaa Ala Val Leu Ser Xaa Leu Pro Lys Glu Glu Gln Ile Gly Lys  
          20                      25                      30  
  
 Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa Arg Arg  
          35                      40

<210> SEQ ID NO 4  
 <211> LENGTH: 38  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived C terminus 38 aa peptide  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (4)..(34)  
 <223> OTHER INFORMATION: X comprises any amino acid  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (37)..(38)  
 <223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 4  
  
 Lys Tyr Tyr Xaa Arg Val Arg Gly Gly Arg Xaa Ala Val Leu Ser Xaa  
 1                      5                      10                      15  
  
 Leu Pro Lys Glu Glu Gln Ile Gly Lys Xaa Ser Thr Arg Gly Arg Lys  
          20                      25                      30

-continued

Xaa Xaa Arg Arg Xaa Xaa  
35

<210> SEQ ID NO 5  
<211> LENGTH: 36  
<212> TYPE: PRT  
<213> ORGANISM: artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: hBD3 derived C terminus 36 aa peptide  
<220> FEATURE:  
<221> NAME/KEY: X  
<222> LOCATION: (2)..(32)  
<223> OTHER INFORMATION: X comprises any amino acid  
<220> FEATURE:  
<221> NAME/KEY: X  
<222> LOCATION: (35)..(36)  
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 5

Tyr Xaa Arg Val Arg Gly Gly Arg Xaa Ala Val Leu Ser Xaa Leu Pro  
1 5 10 15

Lys Glu Glu Gln Ile Gly Lys Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa  
20 25 30

Arg Arg Xaa Xaa  
35

<210> SEQ ID NO 6  
<211> LENGTH: 40  
<212> TYPE: PRT  
<213> ORGANISM: artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: hBD3 derived C terminus 40 aa peptide  
<220> FEATURE:  
<221> NAME/KEY: X  
<222> LOCATION: (6)..(36)  
<223> OTHER INFORMATION: X comprises any amino acid  
<220> FEATURE:  
<221> NAME/KEY: X  
<222> LOCATION: (39)..(40)  
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 6

Leu Gln Lys Tyr Tyr Xaa Arg Val Arg Gly Gly Arg Xaa Ala Val Leu  
1 5 10 15

Ser Xaa Leu Pro Lys Glu Glu Gln Ile Gly Lys Xaa Ser Thr Arg Gly  
20 25 30

Arg Lys Xaa Xaa Arg Arg Xaa Xaa  
35 40

<210> SEQ ID NO 7  
<211> LENGTH: 29  
<212> TYPE: PRT  
<213> ORGANISM: artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: hBD3 derived C terminus 29 aa peptide  
<220> FEATURE:  
<221> NAME/KEY: X  
<222> LOCATION: (2)..(25)  
<223> OTHER INFORMATION: X comprises any amino acid  
<220> FEATURE:  
<221> NAME/KEY: X  
<222> LOCATION: (28)..(29)  
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 7

Arg Xaa Ala Val Leu Ser Xaa Leu Pro Lys Glu Glu Gln Ile Gly Lys  
1 5 10 15

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Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa  
20 25

<210> SEQ ID NO 8  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived C terminus 20 aa peptide  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (8)..(16)  
 <223> OTHER INFORMATION: X comprises any amino acid  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (19)..(20)  
 <223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 8

Lys Glu Glu Gln Ile Gly Lys Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa  
1 5 10 15

Arg Arg Xaa Xaa  
20

<210> SEQ ID NO 9  
 <211> LENGTH: 14  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived C terminus 14 aa peptide  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (2)..(10)  
 <223> OTHER INFORMATION: X comprises any amino acid  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (13)..(14)  
 <223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 9

Lys Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa  
1 5 10

<210> SEQ ID NO 10  
 <211> LENGTH: 19  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived peptide from aa 8 to 26  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (4)..(16)  
 <223> OTHER INFORMATION: X comprises any amino acid

<400> SEQUENCE: 10

Lys Tyr Tyr Xaa Arg Val Arg Gly Gly Arg Xaa Ala Val Leu Ser Xaa  
1 5 10 15

Leu Pro Lys

<210> SEQ ID NO 11  
 <211> LENGTH: 17  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived N terminus 17 aa peptide  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (11)..(11)  
 <223> OTHER INFORMATION: X comprises any amino acid

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&lt;400&gt; SEQUENCE: 11

Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Xaa Arg Val Arg Gly Gly  
 1 5 10 15

Arg

&lt;210&gt; SEQ ID NO 12

&lt;211&gt; LENGTH: 45

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: hBD3 derived peptide, C replaced with W

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: X

&lt;222&gt; LOCATION: (44)..(45)

&lt;223&gt; OTHER INFORMATION: X comprises any amino acid or may be absent

&lt;400&gt; SEQUENCE: 12

Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Trp Arg Val Arg Gly Gly  
 1 5 10 15

Arg Trp Ala Val Leu Ser Trp Leu Pro Lys Glu Glu Gln Ile Gly Lys  
 20 25 30

Trp Ser Thr Arg Gly Arg Lys Trp Trp Arg Arg Xaa Xaa  
 35 40 45

&lt;210&gt; SEQ ID NO 13

&lt;211&gt; LENGTH: 45

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: hBD3 derived peptide, C replaced with F

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: X

&lt;222&gt; LOCATION: (44)..(45)

&lt;223&gt; OTHER INFORMATION: X comprises any amino acid or may be absent

&lt;400&gt; SEQUENCE: 13

Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Phe Arg Val Arg Gly Gly  
 1 5 10 15

Arg Phe Ala Val Leu Ser Phe Leu Pro Lys Glu Glu Gln Ile Gly Lys  
 20 25 30

Phe Ser Thr Arg Gly Arg Lys Phe Phe Arg Arg Xaa Xaa  
 35 40 45

&lt;210&gt; SEQ ID NO 14

&lt;211&gt; LENGTH: 45

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: hBD3 derived peptide, C replaced with Y

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: X

&lt;222&gt; LOCATION: (44)..(45)

&lt;223&gt; OTHER INFORMATION: X comprises any amino acid or may be absent

&lt;400&gt; SEQUENCE: 14

Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Tyr Arg Val Arg Gly Gly  
 1 5 10 15

Arg Tyr Ala Val Leu Ser Tyr Leu Pro Lys Glu Glu Gln Ile Gly Lys  
 20 25 30

Tyr Ser Thr Arg Gly Arg Lys Tyr Tyr Arg Arg Xaa Xaa  
 35 40 45

&lt;210&gt; SEQ ID NO 15

&lt;211&gt; LENGTH: 45

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<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived peptide, C replaced with S
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (44)..(45)
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

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<400> SEQUENCE: 15

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Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Ser Arg Val Arg Gly Gly
1           5           10           15
Arg Ser Ala Val Leu Ser Ser Leu Pro Lys Glu Glu Gln Ile Gly Lys
          20           25           30
Ser Ser Thr Arg Gly Arg Lys Ser Ser Arg Arg Xaa Xaa
          35           40           45

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<210> SEQ ID NO 16
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived peptide, C replaced with A
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (44)..(45)
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

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<400> SEQUENCE: 16

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Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Ala Arg Val Arg Gly Gly
1           5           10           15
Arg Ala Ala Val Leu Ser Ala Leu Pro Lys Glu Glu Gln Ile Gly Lys
          20           25           30
Ala Ser Thr Arg Gly Arg Lys Ala Ala Arg Arg Xaa Xaa
          35           40           45

```

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<210> SEQ ID NO 17
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived peptide, C replaced with C(Acm)
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (11)..(41)
<223> OTHER INFORMATION: X comprises C(Acm)
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (44)..(45)
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

```

```

<400> SEQUENCE: 17

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```

Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Xaa Arg Val Arg Gly Gly
1           5           10           15
Arg Xaa Xaa Val Leu Ser Ala Leu Pro Lys Glu Glu Gln Ile Gly Lys
          20           25           30
Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa
          35           40           45

```

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<210> SEQ ID NO 18
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived peptide, C replaced with C(But)
and terminal KK replaced with XX
<220> FEATURE:

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<221> NAME/KEY: X
<222> LOCATION: (11)..(41)
<223> OTHER INFORMATION: X comprises C(But)
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (44)..(45)
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

```

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<400> SEQUENCE: 18

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Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Xaa Arg Val Arg Gly Gly
1           5           10           15

```

```

Arg Xaa Xaa Val Leu Ser Ala Leu Pro Lys Glu Glu Gln Ile Gly Lys
          20           25           30

```

```

Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa
          35           40           45

```

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<210> SEQ ID NO 19
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived peptide, C replaced with
C(t-Buthio)
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (11)..(41)
<223> OTHER INFORMATION: X comprises C(t-Buthio)
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (44)..(45)
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

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<400> SEQUENCE: 19

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Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Xaa Arg Val Arg Gly Gly
1           5           10           15

```

```

Arg Xaa Xaa Val Leu Ser Ala Leu Pro Lys Glu Glu Gln Ile Gly Lys
          20           25           30

```

```

Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa
          35           40           45

```

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<210> SEQ ID NO 20
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived peptide, C replaced with C(Bzl)
and terminal KK replaced with XX
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (11)..(41)
<223> OTHER INFORMATION: X comprises C(Bzl)
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (44)..(45)
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

```

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<400> SEQUENCE: 20

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Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Xaa Arg Val Arg Gly Gly
1           5           10           15

```

```

Arg Xaa Xaa Val Leu Ser Ala Leu Pro Lys Glu Glu Gln Ile Gly Lys
          20           25           30

```

```

Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa
          35           40           45

```

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<210> SEQ ID NO 21
<211> LENGTH: 45
<212> TYPE: PRT

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<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived peptide, C replaced with
C(4-MeBzl) and terminal KK replaced with XX
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (11)..(41)
<223> OTHER INFORMATION: X comprises C(4-MeBzl)
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (44)..(45)
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

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<400> SEQUENCE: 21

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Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Xaa Arg Val Arg Gly Gly
1           5           10           15

```

```

Arg Xaa Xaa Val Leu Ser Ala Leu Pro Lys Glu Glu Gln Ile Gly Lys
          20           25           30

```

```

Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa
          35           40           45

```

```

<210> SEQ ID NO 22
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived peptide, C replaced with
C(4-MeO-Bzl) and terminal KK replaced with XX
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (11)..(41)
<223> OTHER INFORMATION: X comprises C(4-MeO-Bzl)
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (44)..(45)
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

```

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<400> SEQUENCE: 22

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Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Xaa Arg Val Arg Gly Gly
1           5           10           15

```

```

Arg Xaa Xaa Val Leu Ser Ala Leu Pro Lys Glu Glu Gln Ile Gly Lys
          20           25           30

```

```

Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa
          35           40           45

```

```

<210> SEQ ID NO 23
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived peptide, C replaced with C(Mmt)
and terminal KK replaced with XX
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (11)..(41)
<223> OTHER INFORMATION: X comprises C(Mmt)
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (44)..(45)
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

```

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<400> SEQUENCE: 23

```

```

Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Xaa Arg Val Arg Gly Gly
1           5           10           15

```

```

Arg Xaa Xaa Val Leu Ser Ala Leu Pro Lys Glu Glu Gln Ile Gly Lys
          20           25           30

```

```

Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa
          35           40           45

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<210> SEQ ID NO 24  
 <211> LENGTH: 45  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived peptide, C replaced with any modified C  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (11)..(41)  
 <223> OTHER INFORMATION: X comprises C(Cam), C(But), C(t-Buthio), C(Bzl), C(4-MeBzl), C(4-MeOBzl) or C(Mmt)  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (44)..(45)  
 <223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 24

Gly Ile Ile Asn Thr Leu Gln Lys Tyr Tyr Xaa Arg Val Arg Gly Gly  
 1 5 10 15

Arg Xaa Xaa Val Leu Ser Ala Leu Pro Lys Glu Glu Gln Ile Gly Lys  
 20 25 30

Xaa Ser Thr Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa  
 35 40 45

<210> SEQ ID NO 25  
 <211> LENGTH: 10  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived C terminus 10 aa fragment C replaced with X  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (5)..(6)  
 <223> OTHER INFORMATION: X comprises any amino acid  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (9)..(10)  
 <223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 25

Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa  
 1 5 10

<210> SEQ ID NO 26  
 <211> LENGTH: 10  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived C terminus 10 aa fragment C replaced with W  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (9)..(10)  
 <223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 26

Arg Gly Arg Lys Trp Trp Arg Arg Xaa Xaa  
 1 5 10

<210> SEQ ID NO 27  
 <211> LENGTH: 10  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived C terminus 10 aa fragment C replaced with F  
 <220> FEATURE:

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<221> NAME/KEY: X  
 <222> LOCATION: (9)..(10)  
 <223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 27

Arg Gly Arg Lys Phe Phe Arg Arg Xaa Xaa  
 1 5 10

<210> SEQ ID NO 28  
 <211> LENGTH: 10  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived C terminus 10 aa fragment C  
 replaced with Y  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (9)..(10)  
 <223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 28

Arg Gly Arg Lys Tyr Tyr Arg Arg Xaa Xaa  
 1 5 10

<210> SEQ ID NO 29  
 <211> LENGTH: 10  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived C terminus 10 aa fragment C  
 replaced with L  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (9)..(10)  
 <223> OTHER INFORMATION: X may comprise any amino acid or may be absent

<400> SEQUENCE: 29

Arg Gly Arg Lys Leu Leu Arg Arg Xaa Xaa  
 1 5 10

<210> SEQ ID NO 30  
 <211> LENGTH: 10  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived C terminus 10 aa fragment C  
 replaced with I  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (9)..(10)  
 <223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 30

Arg Gly Arg Lys Ile Ile Arg Arg Xaa Xaa  
 1 5 10

<210> SEQ ID NO 31  
 <211> LENGTH: 10  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 derived C terminus 10 aa fragment C  
 replaced with H  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (9)..(10)  
 <223> OTHER INFORMATION: X comprises any amino acid or may be absent

<400> SEQUENCE: 31

Arg Gly Arg Lys His His Arg Arg Xaa Xaa

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1                      5                      10

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<210> SEQ ID NO 32
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived C terminus 10 aa fragment C
modified
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (5)..(6)
<223> OTHER INFORMATION: X comprises, C(But), C(t-Buthio), C(Bzl),
C(4-MeBzl), C(4-MeOBzl) or C(Mmt)
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (9)..(10)
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

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<400> SEQUENCE: 32

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Arg Gly Arg Lys Xaa Xaa Arg Arg Xaa Xaa
1           5           10

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<210> SEQ ID NO 33
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived C terminus 10 aa fragment C
replaced with V
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (9)..(10)
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

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<400> SEQUENCE: 33

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Arg Gly Arg Lys Val Val Arg Arg Xaa Xaa
1           5           10

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<210> SEQ ID NO 34
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived C terminus 10 aa fragment
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (9)..(10)
<223> OTHER INFORMATION: X comprises any amino acid or may be absent

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<400> SEQUENCE: 34

```

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Arg Gly Arg Lys Cys Cys Arg Arg Xaa Xaa
1           5           10

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<210> SEQ ID NO 35
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived C terminus, C modified
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (5)..(6)
<223> OTHER INFORMATION: C(Acm), C(But), C(t-Buthio), C(Bzl),
C(4-MeBzl), C(4-MeOBzl) or C(Mmt).
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (9)..(10)
<223> OTHER INFORMATION: X may comprise any amino acid or may be absent.

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<400> SEQUENCE: 35

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Arg Gly Arg Lys Xaa Xaa Arg Arg Lys Lys  
1 5 10

<210> SEQ ID NO 36  
 <211> LENGTH: 8  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 repeated fragment excluding terminal KK,  
 C residues replaced with X  
 <220> FEATURE:  
 <221> NAME/KEY: X  
 <222> LOCATION: (5)..(6)  
 <223> OTHER INFORMATION: X comprises any amino acid

<400> SEQUENCE: 36

Arg Gly Arg Lys Xaa Xaa Arg Arg  
1 5

<210> SEQ ID NO 37  
 <211> LENGTH: 8  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 repeated fragment excluding terminal KK,  
 C residues replaced with W

<400> SEQUENCE: 37

Arg Gly Arg Lys Trp Trp Arg Arg  
1 5

<210> SEQ ID NO 38  
 <211> LENGTH: 8  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 repeated fragment excluding terminal KK,  
 C residues replaced with F

<400> SEQUENCE: 38

Arg Gly Arg Lys Phe Phe Arg Arg  
1 5

<210> SEQ ID NO 39  
 <211> LENGTH: 8  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 repeated fragment excluding terminal KK,  
 C residues replaced with Y

<400> SEQUENCE: 39

Arg Gly Arg Lys Tyr Tyr Arg Arg  
1 5

<210> SEQ ID NO 40  
 <211> LENGTH: 8  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: hBD3 repeated fragment excluding terminal KK,  
 C residues replaced with L

<400> SEQUENCE: 40

Arg Gly Arg Lys Leu Leu Arg Arg  
1 5

<210> SEQ ID NO 41

-continued

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<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 repeated fragment excluding terminal KK,
      C residues replaced with I

```

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<400> SEQUENCE: 41

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Arg Gly Arg Lys Ile Ile Arg Arg
1           5

```

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<210> SEQ ID NO 42
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 repeated fragment excluding terminal KK,
      C residues replaced with H

```

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<400> SEQUENCE: 42

```

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Arg Gly Arg Lys His His Arg Arg
1           5

```

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<210> SEQ ID NO 43
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 repeated fragment excluding terminal KK,
      C residues replaced with C(Acm)
<220> FEATURE:
<221> NAME/KEY: X
<222> LOCATION: (5)..(6)
<223> OTHER INFORMATION: C comprises C(Acm), C(But), C(t-Buthio),
      C(Bzl), C(4-MeBzl), C(4-MeOBzl) or C(Mmt).

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<400> SEQUENCE: 43

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Arg Gly Arg Lys Xaa Xaa Arg Arg
1           5

```

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<210> SEQ ID NO 44
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 repeated fragment excluding terminal KK,
      C residues replaced with V

```

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<400> SEQUENCE: 44

```

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Arg Gly Arg Lys Val Val Arg Arg
1           5

```

```

<210> SEQ ID NO 45
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: hBD3 derived peptide, V2 monomer and KK
      residues. 10 aa

```

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<400> SEQUENCE: 45

```

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Arg Gly Arg Lys Val Val Arg Arg Lys Lys
1           5           10

```

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<210> SEQ ID NO 46
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:

```

-continued

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<223> OTHER INFORMATION: V4 monomer, V4-10 AA

&lt;400&gt; SEQUENCE: 46

Arg Gly Arg Lys Val Val Arg Arg Val Val  
 1                      5                      10

&lt;210&gt; SEQ ID NO 47

&lt;211&gt; LENGTH: 10

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Y4 monomer (Y4-10 AA)

&lt;400&gt; SEQUENCE: 47

Arg Gly Arg Lys Tyr Tyr Arg Arg Tyr Tyr  
 1                      5                      10

&lt;210&gt; SEQ ID NO 48

&lt;211&gt; LENGTH: 10

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: W4 monomer, W4-10 AA

&lt;400&gt; SEQUENCE: 48

Arg Gly Arg Lys Trp Trp Arg Arg Trp Trp  
 1                      5                      10

&lt;210&gt; SEQ ID NO 49

&lt;211&gt; LENGTH: 8

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: hBD3 derived peptide, V3 monomer

&lt;400&gt; SEQUENCE: 49

Arg Val Arg Lys Val Val Arg Arg  
 1                      5

&lt;210&gt; SEQ ID NO 50

&lt;211&gt; LENGTH: 8

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: hBD3 derived peptide, V2R monomer

&lt;400&gt; SEQUENCE: 50

Arg Arg Arg Lys Val Val Arg Arg  
 1                      5

&lt;210&gt; SEQ ID NO 51

&lt;211&gt; LENGTH: 8

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: hBD3 derived peptide, V2D monomer

&lt;400&gt; SEQUENCE: 51

Arg Asp Arg Lys Val Val Arg Arg  
 1                      5

&lt;210&gt; SEQ ID NO 52

&lt;211&gt; LENGTH: 8

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: hBD3 derived peptide, E2 monomer

-continued

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<400> SEQUENCE: 52

Arg Gly Arg Lys Glu Glu Arg Arg  
1 5

<210> SEQ ID NO 53

<211> LENGTH: 8

<212> TYPE: PRT

<213> ORGANISM: artificial

<220> FEATURE:

<223> OTHER INFORMATION: hBD3 derived peptide, K2 monomer

<400> SEQUENCE: 53

Arg Gly Arg Lys Lys Lys Arg Arg  
1 5

<210> SEQ ID NO 54

<211> LENGTH: 10

<212> TYPE: PRT

<213> ORGANISM: Artificial

<220> FEATURE:

<223> OTHER INFORMATION: peptide comprising 10 arginines (R)

<400> SEQUENCE: 54

Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg  
1 5 10

<210> SEQ ID NO 55

<211> LENGTH: 4

<212> TYPE: PRT

<213> ORGANISM: artificial

<220> FEATURE:

<223> OTHER INFORMATION: peptide comprising 4 valines (V)

<400> SEQUENCE: 55

Val Val Val Val  
1

<210> SEQ ID NO 56

<211> LENGTH: 4

<212> TYPE: PRT

<213> ORGANISM: artificial

<220> FEATURE:

<223> OTHER INFORMATION: peptide comprising 4 tyrosines (Y)

<400> SEQUENCE: 56

Tyr Tyr Tyr Tyr  
1

<210> SEQ ID NO 57

<211> LENGTH: 8

<212> TYPE: PRT

<213> ORGANISM: artificial

<220> FEATURE:

<223> OTHER INFORMATION: SEQ ID NO: 44 in reverse

<400> SEQUENCE: 57

Arg Arg Val Val Lys Arg Gly Arg  
1 5

<210> SEQ ID NO 58

<211> LENGTH: 9

<212> TYPE: PRT

<213> ORGANISM: artificial

<220> FEATURE:

<223> OTHER INFORMATION: SEQ ID NO: 56 + K

-continued

&lt;400&gt; SEQUENCE: 58

Arg Arg Val Val Lys Arg Gly Arg Lys  
 1 5

&lt;210&gt; SEQ ID NO 59

&lt;211&gt; LENGTH: 8

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

<223> OTHER INFORMATION: hBD3 repeated fragment excluding terminal KK,  
 C residues replaced with V

&lt;400&gt; SEQUENCE: 59

rgrkggrr

8

The invention claimed is:

1. A composition or a combination comprising at least one multimer comprising the formula (U)<sub>2</sub>KK, wherein U comprises SEQ ID NO: 2-53 or 57-59; and at least one other active pharmaceutical ingredient.

2. The composition or combination according to claim 1, wherein U comprises SEQ ID NO: 36.

3. The composition or combination according to claim 1, wherein U comprises SEQ ID NO: 44.

4. The composition or combination according to claim 1, wherein the active pharmaceutical ingredient comprises an antimicrobial compound.

5. The composition or combination according to claim 4, wherein the antimicrobial compound is selected from the group consisting of chloramphenicol, penicillins, macrolides, fluoroquinolones, aminoglycosides,  $\beta$ -lactams or quinolones.

6. The composition or combination according to claim 1, comprising (SEQ ID NO: 44)<sub>2</sub>KK and at least one antimicrobial compound selected from the group consisting of chloramphenicol, penicillins, macrolides, fluoroquinolones, aminoglycosides,  $\beta$ -lactams and quinolones.

7. The composition or combination according to claim 1, comprising (SEQ ID NO: 44)<sub>2</sub>KK and at least one antimicrobial compound selected from the group consisting of carbenicillin, chloramphenicol, erythromycin, gatifloxacin, gentamycin, imipenam, kanamycin, nalidixic acid or streptomycin.

8. The composition or combination according to claim 1, comprising (SEQ ID NO: 44)<sub>2</sub>KK and gatifloxacin.

9. The composition or combination according to claim 1, wherein the multimer and the antimicrobial compound acts synergistically.

10. A method of treating at least one microbial infection comprising administering to a subject at least one multimer comprising the formula (U)<sub>2</sub>KK, wherein U comprises SEQ ID NO: 2-53 or 57-59; and at least one other active pharmaceutical ingredient.

11. The method according to claim 10, wherein U comprises SEQ ID NO: 36.

12. The method according to claim 10, wherein U comprises SEQ ID NO: 44.

13. The method according to claim 10, wherein the active pharmaceutical ingredient comprises an antimicrobial compound.

14. The method according to claim 10, wherein U comprises SEQ ID NO: 44 and the antimicrobial compound comprises carbenicillin, chloramphenicol, erythromycin, gatifloxacin, gentamycin, imipenam, kanamycin, nalidixic acid or streptomycin.

15. The method according to claim 11, wherein the multimer and the active pharmaceutical ingredient are administered separately.

16. The method according to claim 11, wherein the multimer and the active ingredient are administered together.

17. The method according to claim 10, wherein the multimer and the active pharmaceutical ingredient act synergistically.

18. A method of inhibiting and/or reducing the growth of microorganisms comprising contacting at least one microorganism with at least one isolated multimer comprising the formula (U)<sub>2</sub>KK, wherein U comprises SEQ ID NO: 2-53 or 57-59; and at least one other antimicrobial compound.

\* \* \* \* \*



UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 9,220,264 B2  
APPLICATION NO. : 14/090057  
DATED : December 29, 2015  
INVENTOR(S) : Beuerman et al.

Page 1 of 1

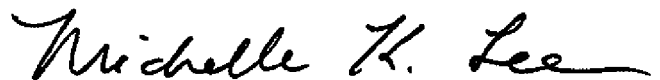
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims:

Column 70, Line 37: Claim 15, Delete "Claim 11" and insert --Claim 10--

Column 70, Line 40: Claim 16, Delete "Claim 11" and insert --Claim 10--

Signed and Sealed this  
Twenty-third Day of August, 2016

A handwritten signature in black ink, reading "Michelle K. Lee". The signature is written in a cursive, flowing style.

Michelle K. Lee  
*Director of the United States Patent and Trademark Office*